IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Alan Gewirtz** Confirmation No.: **6995**

Serial No.: **09/993,183** Group Art Unit: **1645**

Filing Date: **November 14, 2001** Examiner: **Kimberly Chong**

For: **Post-Transitional Gene Silencing by RNAi in Mammalian Cells**

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

APPELLANT'S BRIEF PURSUANT TO 37 C.F.R. § 41.37

This brief is filed in support of Appellant's appeal from the rejections of claims 1, 2, 5, 7-9, 11, and 21-38, dated March 30, 2007. A Notice of Appeal was timely filed on July 2, 2007.

1. REAL PARTY IN INTEREST

The real party in interest is the assignee of the patent application, The Trustees of the University of Pennsylvania. To ensure full compliance with the spirit of 37 C.F.R. § 41.37(c)(1), Appellant notes that the subject application's exclusive licensee, Acuity Pharmaceuticals, Inc., now wholly-owned by Opko Corp., may also have an interest in the outcome.

2. RELATED APPEALS AND INTERFERENCES

None of the Appellant, Appellant's Assignee, or Appellant's undersigned representative are presently aware of any prior or pending appeals, interferences, or judicial proceedings that are related to, will directly affect, or will have a bearing on the Board's decision in this appeal.

3. STATUS OF CLAIMS

Claims 1, 2, 5, 7-9, 11, and 21-38 are currently pending, and each stands finally rejected. The rejection of claims 1, 2, 5, 7-9, 11, and 21-38 is appealed.

4. STATUS OF AMENDMENTS

All amendments that have been submitted during prosecution have been entered. No amendments have been submitted subsequent to the final rejection mailed March 30, 2007.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The claims at issue are directed to methods for disrupting expression of a mammalian target gene by RNA interference (RNAi). Of the appealed claims, claims 1, 22, and 28 are independent.

Claim 1 is directed to a method for disrupting expression of a mammalian target gene in a human cell. (Specification, page 5, lines 15-18). The method comprises initiating RNA interference (RNAi) *in vitro* by exposing a human cell (e.g., page 8, line 4) to a double-stranded RNA (dsRNA) (page 9, lines 12-28) homologous to the target gene (page 8, lines 10-17). The dsRNA consists essentially of two complementary linearized strands of RNA, wherein the transcription of each of the complementary strands is independently controlled to generate paired RNAs of defined length (page 9, lines 12-28; see also Examples, page 12, line 28 through page 13, line 12).

Claim 22 is directed to a method for disrupting expression of a mammalian target gene in a human cell *in vitro*. The method comprises providing an RNA sequence homologous to a portion of the target gene, wherein the RNA is capable of inducing RNAi of the target gene. (See, e.g., "Examples", page 12, lines 22-27 through page 14, line 25).

Claim 28 is directed to a method for disrupting expression of a target gene in a human cell. The method comprises the steps of: selecting a human cell expressing the target gene (e.g., page 12, lines 23-27, page 8, lines 11-13, and page 13, lines 13-14); preparing a double-stranded RNA (dsRNA) consisting essentially of a first strand homologous to the target gene, and a second strand complementary to the first strand (page 9, lines 12-28, and page 12, line 28 through page 13, line 12); exposing the human cell to the dsRNA in a reaction mixture *in vitro* (page 13, line 16-17), under conditions permitting the dsRNA to enter the cell (page 13, line 16-18, and page 13, line 28 through page 14, line 16), and incubating the reaction

mixture for a time sufficient to allow the initiation of RNA interference (page 13, line 16-18, and page 13, line 28 through page 14, line 16), whereby the expression of the target gene is disrupted (page 13, line 27 through page 14, line 25, see also Table 1) .

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The grounds of rejection to be reviewed on appeal are:

Whether claims 1, 2, 5, 7-9, 11, 21-22, and 24-27 are patentable under 35 U.S.C. § 102 (b) in view of Fire *et al.* (U.S. Patent No. 6,506,559) ("Fire").

Whether claims 1, 2, 5, 7-9, 11 and 21-38 are patentable under 35 U.S.C. § 103 (a) over Fire in view of Kreutzer *et al.,* WO 00/44895 ("Kreutzer"), Gewirtz *et al.,* WO 92/19252 ("Gewirtz"), and Sharp, *Genes & Dev.* 13:139-141, 1999.

7. ARGUMENT

Summary of Argument

The rejection of claims 1, 2, 5, 7-9, 11, 21-22, and 24-27 under 35 U.S.C. § 102 (b) should be reversed. The rejection is premised on vague and broadly generalized language in the allegedly anticipatory Fire patent. Fire is not novelty-destroying under 35 U.S.C. § 102 because the reference did not enable a skilled artisan to practice Appellant's claimed invention at the time of Appellant's filing. At that time, the skilled artisan would not have concluded that Fire's teachings were applicable to mammalian cells, based on statements incorporated by reference into Fire itself, and based on the state of the art as evidenced by peer-reviewed publications authored by the Fire patent inventors, as well as publications by others.

The rejection of claims 1, 2, 5, 7-9, 11 and 21-38 under 35 U.S.C. § 103 (a) over Fire and secondary references should also be reversed. Kreutzer is not available as prior art and, even if it was, the asserted references in combination do not disclose all the features of Appellant's claimed invention. The rejection must also be reversed because the Examiner erred in disregarding the teachings away in the references themselves, and in failing to consider the references and Appellant's claimed invention as a whole.

I. The Claims are Novel Over Fire

Appellant respectfully submits that the claims are novel over Fire and that the rejection should be reversed because Fire did not enable the practice of Appellant's invention. Thus, Fire is not novelty-destroying under 35 U.S.C. § 102 (b).

A. The Legal Standard for Anticipation

Under 35 U.S.C. § 102 (b), a claim is anticipated only when each and every element in the claim is found, either expressly or inherently, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co.*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The same invention, with all the limitations of the claims, must have existed in the prior art. *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920-21 (Fed. Cir. 1989) ("anticipation" requires that the identical invention is described in a single prior art reference). The Manual for Patent Examination Procedure ("MPEP") states "The identical invention must be shown in as complete detail as is contained in the ... claim." MPEP 2131.01 citing *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989).

An anticipatory reference must enable one of ordinary skill in the art to make and use the invention. *Bristol-Myers Squibb Co. v. Ben Venue Labs. Inc.*, 246 F.3d 1368, 1378, 58 USPQ 2d 1508, 1516 (Fed. Cir. 2001). What a prior art reference actually discloses for purposes of anticipation is a factual determination. *Tegal Corp. v. Tokyo Electron Am., Inc.*, 257 F.3d 1331, 1338–39 (Fed. Cir. 2001). However, whether a prior art reference is enabling is a question of law based upon underlying factual findings. *SmithKline Beecham Corp. v. Apotex Corp.*, 403 F.3d 1331, 1337, 74 USPQ2d 1398 (Fed. Cir. 2005). As MPEP 2121.01 states: "In determining that quantum of prior art disclosure which is necessary to declare an applicant's invention 'not novel' or 'anticipated' within section 102, the stated test is whether a reference contains an 'enabling disclosure'. . .. *In re Hoeksema*, 399 F.2d 269, 158 USPQ 596 (CCPA 1968)." Once a reference is alleged to contain all of the elements of the claimed invention, the reference is presumed to be enabling, and the burden shifts to the applicant to provide facts rebutting the presumption. *In re Sasse*, 629 F.2d 675, 207 USPQ 107 (CCPA 1980).

"A claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled." *Amgen, Inc. v. Hoechst Marion*

Roussel, Inc., 314 F. 3d 1313, 1354 (Fed. Cir. 2003). The reference must contain sufficient disclosure "to enable one of skill in the art to reduce the disclosed invention to practice." *Id.* Thus, even if the claimed invention is disclosed in a printed publication, that disclosure will not suffice as prior art if it was not enabling. *In re Borst*, 345 F.2d 851,855, 145 USPQ 554, 557 (CCPA 1965) (*cert. denied*).

The Supreme Court in *Seymour v. Osbourne*, 78 U.S. 516 (1870) observed:

> Patented inventions cannot be superceded by the mere introduction of a [prior art reference] unless the description and drawings contain and exhibit a substantial representation of the patented improvement, in such full, clear, and exact terms as to enable any person skilled in the art of science to which it appertains, to make, construct, and practice the invention to the same practical extent as they would be enabled to do if the information was derived from a prior patent. **Mere vague and general representations will not support such a defense, as the knowledge supposed to be derived from the publication must be sufficient to enable those skilled in the art or science to understand the nature and operation of the invention, and carry it into practice use.** Whatever may be the particular circumstances under which the publication takes place, the account published, to be of any effect to support such a defense, must be an account of complete and operative invention capable of being put into practical operation.

78 U.S. 516, 555 (emphasis added).

In *Elan Pharm. Inc. v. Mayo Found. For Med. Educ. & Research*, 346 F.3d 1051, 1054, 68 USPQ2d 1373 (Fed. Cir. 2000), the Federal Circuit held that to serve as an anticipating reference, the reference must enable the allegedly anticipated subject matter.

The challenged reference in *Elan* was a U.S. patent to Mullan that, like Fire here, disclosed a biological method. The *Elan* court said

> The issue is not whether the teachings are an accurate compilation of the scientific state of the art at that time, and they are not challenged on that ground. The issue is whether his [Mullan's] teachings enabled a person of ordinary skill, without undue experimentation, to produce the desired transgenic mouse.

(*Elan*, 346 F.3d at 1057).

Prior cases framed the enablement inquiry differently, stating that to be anticipatory, a prior art reference must place the public in possession of the invention. See *In re Donohue*, 766 F.2d 531, 226 USPQ 619 (Fed. Cir. 1985); see also *In re Borst*, 345 F.2d 851,855, 145 USPQ 554, 557 (CCPA 1962).

In *Elan* (346 F.3d at 1056-57), the court stated "an enablement determination is made retrospectively, i.e., by looking back to the filing date of the patent application and

determining whether undue experimentation would have been required to make and use the claimed invention at that time" based on the allegedly anticipatory reference.

B. The Anticipation Rejection and Fire

Claims 1, 2, 5, 7-9, 11, 21-22, and 24-27 stand rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by Fire *et al.* (U.S. Patent No. 6,506,559) ("Fire"). Fire was filed December 18, 1998, and issued January 14, 2003. Fire claims priority to a U.S. provisional patent application filed December 23, 1997. A copy of Fire is attached hereto as Exhibit 10 in Evidence Appendix.

The Examiner alleged that Fire teaches a method for inhibiting expression of a target gene using double-stranded RNA (dsRNA) to induce RNAi in a cell *in vitro* in an animal, citing Fire's claims 1 and 6 (at column 26) as support.

Claims 1 and 6 of Fire, cited in the rejection under 35 U.S.C. § 102 (b), recite respectively:

> 1. A method to inhibit expression of a target gene in a cell *in vitro* comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule and the double- stranded molecule inhibits expression of the target gene.
>
> and
>
> 6. The method of claim 1 in which the cell is from an animal.

The Examiner further asserted that Fire, at column 8, lines 13-14, discloses that the cell with the target gene may be derived from or contained within any organism.

The cited (italicized) portion of Fire presented in the context of the surrounding disclosure, reads as follows:

Column 8, lines 13-14:

The cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Fire, column 8, lines 35-37, was cited to establish that examples of vertebrate organisms include mammals and humans. The cited portion of Fire is shown in italics below in context of the surrounding disclosure.

Column 8, lines 35-37:

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, . . .

The rejection also cited Fire, column 8, lines 52-55, as stating that the target gene may be "immortalized or transformed, or the like." The cited portions are shown in italics below in the context of the surrounding disclosure.

Column 8, lines 52-55:

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, . . .

Column 10, lines 26-28, of Fire was cited to indicate that Fire's invention "could be used for treatment or development of treatments for cancer of any type, including solid tumors and leukemias." Again, the cited portion is shown in context below:

Column 10, lines 26-28:

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome,

carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mutinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia. . .

The rejection asserted that Fire discloses that lipid-mediated carrier transport can be used to introduce nucleic acids to cells (Fire, column 9, lines 55-60). In context, this portion of Fire recites the following:

Column 9, lines 55-60:

Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or otherwise increase inhibition of the target gene.

The Examiner alleged that Fire (column 6, lines 55-57) discloses that "inhibition of gene expression" refers to the absence or observable decrease in the level of protein and/or mRNA product from a target gene, thereby indicating disruption of gene function. The cited portion states:

Column 6, lines 55-57:

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene.

Appellant respectfully submits that the cited portions of Fire relied on in the rejection, when viewed in context of the surrounding disclosure, are entirely "**vague and general representations**" of the type the Supreme Court held in *Seymour* were not sufficient to anticipate.

Fire generally describes a dsRNA inhibition phenomenon in invertebrate animals, such as the worm, *Caenorhabditis elegans* (column 14, line 41 through column 22, line 4). Specifically, Fire teaches introducing dsRNA into *C. elegans* via injection (e.g., column 17, line 25-42, and Figure 3), via feeding (column 17, lines 43-49, and Table 3), or via bathing larvae in a solution containing the dsRNA (column 18, line 20-41, and Table 4). By doing so, Fire was able to show for the first time that the double-stranded structure of the RNA was important to the mechanism of RNAi in *C. elegans*, as compared to antisense or sense-strand inhibition (column 14, line 56 through column 15, line 27).

As established via evidence of record summarized below, from the time of Fire's filing, up through the time of Appellant's filing, Fire did not enable a skilled artisan to practice Appellant's claimed invention. In particular, Fire did not enable the practice of the claimed invention in any mammals, including humans, because Fire was not a "complete and operative invention capable of being put into practical operation" for mammalian systems. (see *Seymour, supra*). The cited disclosures from Fire, when properly viewed in the "vague and general" context in which they were made, would not have enabled the ordinarily skilled artisan to practice Appellant's claimed invention.

C. The Teachings of the Related Art

The discovery of RNA-mediated target gene inhibition in plant and invertebrate animal systems created intense scientific curiosity about whether such mechanisms existed in vertebrate and particularly, mammalian systems. (see *e.g.*, Gewirtz declaration, of record, discussed *infra*). Prior to the discoveries disclosed in Appellant's specification, it was neither known nor believed by artisans skilled in the field, that vertebrate animals, and more particularly mammals, possessed the cellular machinery or pathways that would be necessary or sufficient to allow RNAi to operate in a useful manner.

The relevant art, from at least Fire's filing date through Appellant's filing date, was replete with peer-reviewed papers that indicated that RNAi could not be successfully practiced in vertebrate animal or mammalian systems. According to the literature of the period, either the mechanisms for RNAi did not exist in vertebrate systems, or alternatively, even if mechanisms for RNAi did exist in vertebrates, cellular defense mechanisms of vertebrate cells would preclude the use of such mechanisms for selective gene silencing. It was widely considered that generalized cellular responses to foreign RNA in vertebrate systems, such as the antiviral "panic" responses, including the predominant dsRNA-responsive phosphokinase ("PKR") response, would override any attempts at intentional introduction of dsRNA in such cells to cause RNA interference. The cellular events resulting from introduction of dsRNA would lead to drastic negative consequences for the cell, including general translational arrest, and apoptosis (cell death).

The record evidence is this regards is summarized as follows:

1. Dr. Fire's own work, published in *Trends in Genetics*, 15:358-363 (1999) ("Fire *TiG*") (Exhibit 1, Evidence Appendix), published *after the filing date* of Fire, establishes that

not only Dr. Fire, but *skilled artisans generally*, had no knowledge that RNAi could be practiced in mammals, and certainly did not believe the "simple protocols" used for invertebrates and plants would be successful in mammalian or human cells. Fire *TiG* stated, for example:

> From a technical perspective, one could certainly hope that RNA-triggered silencing would exist in vertebrates: this would facilitate functional genomics and might allow medical applications involving targeted silencing of 'renegade' genes. Although this hope is not ruled out by any current data, the simple protocols used for invertebrate and plant systems are unlikely to be effective.

Dr. Fire thus himself admitted that the method disclosed in his patent, necessary to the practice of the invention, did not yet exist for vertebrates at the time Fire *TiG* was published. Notwithstanding his patent disclosure, Dr. Fire himself did not embrace the applicability of his method for mammalian cells. Despite references to carrying out RNAi in "animals" in the Fire patent, the applicability of the Fire invention, by the inventor's own admission, was limited to the invertebrate animals.

There is no reasonable interpretation of the Fire *TiG* publication that supports the Examiner's contention that the Fire patent enabled the skilled artisan at the time to practice the invention now claimed by Appellant.

2. Montgomery and Fire, *Trends in Genetics* 14(7):255-257 (1998) ("Montgomery and Fire"), (Exhibit 2, Evidence Appendix). reveals the prevailing state of the art at the relevant time. Montgomery and Fire provides compelling evidence that the Fire patent did not enable the skilled artisan to practice Appellant's claimed invention. Authored by Dr. Fire and co-inventor Dr. Mary Montgomery, the article contains a section entitled "Do RNA-interference mechanisms have counterparts outside of plants and nematodes?" The article did not purport to answer the question posed, thus, the skilled artisan could only conclude from Montgomery and Fire that it was not known at the time whether RNAi even existed in mammals.

After reviewing the art, including the "global antiviral response" of mammalian cells to dsRNA, the "PKR response", and the "general translational arrest" that were appreciated to occur in mammalian cells exposed to dsRNA, Montgomery and Fire suggested that, because such responses can occur even where the dsRNA is taken up after being provided

extracellularly, those responses probably evolved from responses to viral challenges. Montgomery and Fire conclude that:

> Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of activating PKR.

The above-statement is *incorporated by reference* in Fire, and thus **constitutes a teaching of the Fire patent itself**. See Fire's specification, column 18, lines 42-46, and column 22, lines 5-8. Fire states that the references cited therein are indicative of the level of skill in the art at the time.

Thus, Fire discusses the state of the art at the time. At most, it was known in the art that ***if RNAi existed in such cells***, it would **probably be necessary** either to **induce a transient lapse in the PKR response**, or **to use a dose incapable of activating the PKR response** to practice RNAi methods in mammalian cells. Yet, Fire provides no guidance on any methods or means of inducing a transient lapse in the PKR response, nor were any known in the art or predictable at the time of Appellant's filing. There is no teaching nor does Fire provide any guidance whatsoever as to any conditions, doses, or the like, that might be helpful or useful in achieving such a "transient lapse in the PKR response." Nor is there any guidance in Fire as to doses that might provide a "controlled level of dsRNA" incapable of activating the PKR response. There is no guidance on doses that are capable of inducing RNAi in any vertebrate cell, regardless of whether such doses activate the PKR response.

3. Sharp (Exhibit 3, Evidence Appendix), is evidence of the state of the art just after the filing date of Fire. Sharp was cited by the Examiner as allegedly supporting the proposition that RNAi is "a general mechanism for gene regulation and may be critical for many developmental and antiviral processes." The Examiner has taken Sharp out of context. The quote is found in the introductory paragraph of the paper, immediately following a list of all the organisms in which dsRNA silencing of genes was known. The list is limited to the invertebrates, *C. elegans*, *Trypansoma brucei,* and *Drosophila*, and plants. The quoted language must be viewed in its proper context within the entire paper focusing on invertebrates. In the only paragraph in the three-page paper to address dsRNA in vertebrates, Sharp concluded that "[p]erhaps some aspect of the RNAi effect occurs or can be induced in mammalian cells."

As a whole, Sharp suggested that, as of its 1999 publication date, RNAi was an established phenomenon in invertebrates, and that dsRNA-mediated suppression of specific genes was known in plants. But Sharp also indicated that it was not known whether any aspect of RNAi occurred in, or could be induced in, mammalian cells. Accordingly, the skilled artisan could not have practiced Appellant's claimed invention based on the disclosure in Fire and the state of knowledge existing in the art at the time.

4. Kreutzer's patent application, U.S. Patent Application Publication 2004/0175703, published September 9, 2004 ("Kreutzer Patent Application") (Exhibit 4, Evidence Appendix), described the state of the art. The Kreutzer Patent Application reflects a considered conclusion that protocols used for invertebrate and plant systems would not be effective for mammalian applications of dsRNA:

> WO 99/32619 (Fire *et al.*) discloses the use of a dsRNA of at least 25 nucleotides in length to inhibit the expression of a target gene in *C. elegans*. dsRNA has also been shown to degrade target RNA in other organisms, including plants (see, *e.g.*, WO 99/53050, Waterhouse et al.; and WO 99/6163-1, Heifetz *et al.*) and *Drosophilia* (see, *e.g.*, Yang, D., *et al., Curr. Biol.* (2000) 10:1191-1200). Despite successes in these organisms, until recently the general perception in the art has been that RNAi cannot be made to work in mammals. It was believed that protocols used for invertebrate and plant systems would not be effective in mammals due to the interferon response, which leads to an overall block to translation and the onset of apoptosis (see, *e.g.*, Wianny, F., *et al., Nature Cell Biol.* (2000) 2:70-75; Fire, A., *Trends Genet.* (1999) 15:358-363; and Tuschl, T., *et al., Genes Dev.* (1999) 13(24):3191-97). At least one group of scientists believed that RNAi could only be made to work in mammals if the PKR response could be neutralized or some way avoided, although no suggestions were given as to how this might be achieved (Fire, *Trends Genet* (1999), *supra*; and Montgomery and Fire, *Trends Genet.* (1998) 14:255-258). However, WO 00/44895 (Limmer) demonstrated for the first time that dsRNA can induce RNAi in mammalian cells, provided that the dsRNA meets certain structural requirements, including a defined length limitation.
>
> Kreutzer Patent Application (see paragraph [0006])

The discussion in Kreutzer Patent Application is consistent with Appellant's position that Fire did not enable the skilled artisan at the time of Appellant's filing to practice the invention now claimed by Appellant. Although this discussion did not appear in a peer-reviewed journal article, the disclosure nonetheless constitutes part of the relevant art (see, e.g., MPEP 2123) and must be given fair weight as evidence. While Kreutzer Patent

Application itself is not prior art to the Appellant's invention having been published in 2004, the quoted segment is part of the background section of the Kreutzer Patent Application, and is amply referenced with citations to the peer-reviewed literature that accurately reflect the state of the art during the period from Fire's filing up to about the time of Appellant's filing. Many of these references are already of record in the instant prosecution history.

5. A peer-reviewed paper by Paddison *et al., Proc. Nat'l Acad. Sci.* 99(3):1443-1448 (Feb. 5, 2002) ("Paddison"), attached hereto as Exhibit 5 (Evidence Appendix), has been cited by Appellant during prosecution as further evidence that Fire does not enable the skilled artisan to practice Appellant's claimed invention. Paddison teaches that "[i]t has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans." Citing the work of Wianny *et al.* (*Nat. Cell Biol.* 2:70-75, 2000) ("Wianny"), as well as that of Svoboda *et al.* (*Development (Cambridge UK)* 127: 4147-4156, 2000) ("Svoboda"), Paddison stated in his discussion that "[t]he first indication that this (RNAi) response might also extend to mammals came from the observation that injection of dsRNAs into early mouse embryos induced sequence specific silencing". Appellant notes that Paddison was published in one of the most prestigious scientific journals in the United States. Further, the work of Wianny and Svoboda on gene silencing in early mouse embryos published three years after the filing of the Fire patent.

6. Wianny (Exhibit 6, Evidence Appendix) and Svoboda (Exhibit 7, Evidence Appendix), both peer-reviewed research articles, provide further evidence that Fire's disclosure was insufficient to enable the ordinarily skilled artisan to practice Appellant's claimed invention. Wianny stated that "[s]o far, there has been no report that RNAi can be used in mammals. Moreover there are several indications of potential limitations to its function in the group of animals." As of Wianny's publication date, RNAi in mammalian cells had only been accomplished in early embryonic cells of mouse. These cells are not directly relevant to the practice of RNAi in mammalian cells generally, because the early embryonic cells used by Wianny were known or believed to lack the RNA-defense mechanisms that the entire art recognized as problematic for the potential practice of RNAi in mammalian systems. At most, Wianny's work may have demonstrated that the underlying mechanisms of RNAi may exist in mammalian cells (at least at the earliest stage developmentally), but it did not solve the problem regarding the practice of RNAi in

developed cells, i.e., those having the cellular defense mechanisms against introduced RNA, such as the PKR response.

7. Finally, the declaration submitted during prosecution by Appellant Dr. Alan Gewirtz provides evidence that a skilled artisan would not have been able to practice Appellant's claimed invention based on Fire and the knowledge in the art at the time of Fire's filing. Evidence that Dr. Fire, a skilled artisan in the field of RNAi, admitted that the methods he disclosed could not be practiced in mammalian cells, or more specifically, human cells even after the Fire patent was filed, was also summarized in Dr. Gewirtz's declaration. Dr. Gewirtz also addressed many of the publications discussed herein and cited during prosecution. Dr. Gewirtz's declaration, dated September 14, 2005, was submitted with the response filed September 16, 2005 to the Office Action mailed May 23, 2005, and a copy is attached hereto as Exhibit 8 of the Evidence Appendix.

D. Fire Does Not Anticipate Appellant's Claimed Invention Since it Does Not Teach and Did Not Enable the Skilled Artisan to Practice the Claimed Invention

Fire does not anticipate the claimed invention at least because the prior art, as evidenced by the foregoing citations, could not supply the information lacking in Fire to allow the skilled artisan to practice Appellant's invention. It was neither known nor believed that the mechanisms and cellular machinery for RNAi existed in mammals, especially humans. Fire did not teach how to practice RNAi in any vertebrate or mammalian systems or cells. Further, Fire and his coinventor Montgomery, by their own contemporaneous and post-filing writings, acknowledged that the state of the art was such that RNAi could not be practiced in mammalian systems because of the various RNA defense mechanisms.

Fire was not an enabling disclosure for RNAi in mammalian cells as of Appellant's filing date. The evidence of record, in the form of peer-reviewed papers, patent publications, and an affidavit from the inventor, himself a skilled artisan, has established that according to the state of the art, from the time of Fire's filing and up to and including the time of Appellant's filing, Appellant's claimed invention was neither achieved nor achievable prior to Appellant's filing date. The Examiner has erred in giving weight to generalized "teachings" and vague "boilerplate" language in Fire that are insufficient to rise to the level of enabling disclosure to qualify as anticipatory against Appellant's claimed invention.

Appellant's evidence is more than sufficient to overcome any presumption of enablement by Fire.

In rebuttal, the Examiner cited Kreutzer, a reference which has been antedated during the instant prosecution (see discussion below under § 103 rejections), as evidence of the enablement of RNAi in mammalian cells. The Examiner has relied on unspecified "post-filing date art", which allegedly "has repeatedly shown that the methods of Fire *et al.* work in human cells." (see Office Action of March 30, 2007, page 10.) Such unspecified art was used as "additional support that the methods taught by Fire *et al.* anticipate the instantly claimed invention." *Id.* The Examiner has declined Appellant's invitation to provide specific examples of art with an effective date after Fire's filing, but which pre-dates Appellant's filing.

Further, the Examiner has applied an incorrect legal standard for determining enablement by Fire, and thus, anticipation by Fire. The Examiner stated:

> The enablement requirement for prior art to anticipate under section 102 is different than the enablement requirement required to anticipate under section 112 and this fact is made clear by the recent decision by the Federal Circuit in *Impax Labs, Inc. v. Aventis Pharm.* (Fed. Cir. 2006) cited by applicant. Here the Federal Circuit stated "[t]he standard for enablement of a prior art reference for purposes of anticipation under section 102 differs from the enablement standard under 35 U.S.C. § 112. . .While section 112 provides that [t]he specification must enable one skilled in the art to "use" the invention,...section 102 makes no such requirement as to an anticipatory disclosure..."
>
> Therefore, because an issued patent is presumed enabled, and because applicants have not shown any manipulative differences or shown any structural differences in the steps used in the instantly disclosed methods as compared to the methods disclosed by Fire *et al.*, the methods taught by Fire *et al.* anticipate the claimed invention.

Office Action mailed March 30, 2007, page 12.

The Examiner may have correctly observed that the legal standard for enablement under 35 U.S.C. § 102 is different from that under 35 U.S.C. § 112, however, a proper legal standard was neither stated nor applied by the Examiner. The cited language from *Impax* does not provide an adequate legal standard for the determination of the issue of enablement presented here. The Examiner's reliance on language in *Impax* distinguishing "making" from "using" is misplaced. Fire taught neither how to "make" nor how to "use" methods of RNAi in vertebrate systems. Fire simply would not have enabled the skilled artisan to practice the claimed invention.

The law requires that an allegedly anticipatory reference enable the claimed invention. *Elan (*346 F.3d at 1054, citing *Amgen*, and stating that "[e]nablement requires that the prior art reference must teach one of ordinary skill in the art to make or carry out the invention without undue experimentation") Whether a prior art reference is enabling is a question of law based upon underlying factual findings. *SmithKline Beecham* (*supra*). Thus, a proper legal standard must be applied by the Examiner, and a proper factual analysis must underlie the ultimate legal conclusion. The Examiner's reliance on statements by the divided panel in *Impax* ignores the holdings set forth in *Impax* itself, as well as the law of both *Elan* and *Amgen, supra*. Those cases, as well as the Supreme Court's decision in *Seymour*, state that to anticipate, a reference ***must enable a skilled artisan to practice the invention against which the reference is asserted***. Here, Fire did not enable the skilled artisan to practice Appellant's claimed invention.

Unlike *Impax*, which involved questions of efficacy of fully disclosed compounds for methods of treatment, no such claims or issues are present here. The portion of *Impax* relied on by the Examiner is taken out of context. *Impax* held that the "proper issue is whether the [prior art reference] is enabling in the sense that it describes the claimed invention sufficiently to enable a person of ordinary skill in the art to carry out the invention." *Impax Labs, Inc. v. Aventis Pharm.*, 468 F.3d 1366 (Fed. Cir. 2006) (vacating the district court's finding of anticipation and remanding to determine whether the reference "is enabling in that the person of ordinary skill can carry out the invention").

The issue here is whether Fire disclosed Appellant's claimed invention in an anticipatory manner, i.e., in a manner that would have enabled a skilled artisan to practice the steps of those methods at the time of Appellant's filing Proper consideration of enablement of a prior art reference requires factual analysis under *In re Wands* (858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988)) to determine whether a skilled artisan could practice the claimed invention without undue experimentation. *Elan,* 346 F.3d at 1055 (citing *In re Goodman* (11 F. 3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993) as an example of the factual analysis required for determining enablement of a method of gene manipulation). The enablement of Fire must thus be assessed starting with a factual analysis under *Wands* to determine whether the skilled artisan could practice the instantly claimed invention using only Fire and the knowledge in the art at the time.

Analysis under *Wands* requires consideration of whether or not one of ordinary skill in the art can make and use an invention ***without undue experimentation***. The *Wands* court

explained that enablement analysis for biological processes required factual assessment of whether the requisite experimentation to practice a claimed invention is ***undue***. The factual assessment may include, but is not limited to, consideration of the so-called "*Wands* factors":

(1) the quantity of experimentation necessary to practice the claimed invention,

(2) the amount of direction or guidance presented,

(3) the presence or absence of working examples,

(4) the nature of the invention,

(5) the state of the prior art,

(6) the relative skill of those in the art,

(7) the predictability or unpredictability of the art, and

(8) the breadth of the claims at issue.

Wands, 858 F.2d at 737.

The ***facts*** of Appellant's case are very similar to those of *Goodman.* 11 F. 3d at 1052, 29 USPQ2d at 2015. Although *Goodman* deals with enablement of claims under § 112, and not enablement of a prior art reference under § 102, the ***required factual analysis*** under *Wands* is indistinguishable. Given the parallels with Appellant's case, *Goodman's* underlying ***factual analysis*** is instructive.

Goodman claimed a method of manufacturing mammalian peptides in plant cells using *Agrobacterium*-mediated transformation. Goodman's specification *exemplified* only dicots. 11 F. 3d at 1051, 29 USPQ2d at 2015. The Federal Circuit upheld the Board's rejection of the claims for lack of enablement because the specification did not contain sufficient information to enable the scope of the claims beyond dicots. The court noted, for example, that the production of peptides in monocot plants involves extensive problems unaddressed by Goodman's specification. *Id.*

Notably, the *Goodman* court was persuaded to uphold the enablement rejection there in view of the unpredictability of the art, which the court found reflected in an article stating:

> It has been widely considered that monocotyledonous plants, including the commercially important crop plants of the *Gramineae* family, are insensitive to [Ti plasmid transformation] and thus are not candidates for the use of this gene transfer system. Two more recent reports have modified this opinion to some extent. . .

Goodman, 11 F. 3d at 1051.

The court concluded that this article, coupled with the "hedgings" in other references, showed unpredictability in the art when Goodman filed his application in 1985. The court relied on yet another paper, published by Goodman himself two years after his filing date, in which he had reported only limited success. Goodman's paper stated that "[i]n plants, viral-based vectors are not likely to stably transform plant cells because integration of viral genes into plant chromosomes has not been detected." *Id.* at 1051-52. As a result of the unpredictability of the art and Goodman's admission, the Federal Circuit held:

> Thus, on Goodman's 1985 filing date, the record shows no reliable gene transformation method for use with monocot plants. Each of the methods for monocot plants was fraught with unpredictability. The teachings in the specification do not cure this unpredictability. The record shows that practicing a gene transformation method for all monocot plants, if possible at all in 1985, would have required extensive experimentation that would preclude patentability. [**16] See *White Consol. Indus. Inc. v. Vega Servo-Control, Inc.*, 713 F.2d 788, 218 U.S.P.Q. (BNA) 961 (Fed. Cir. 1983). . . .
>
> The record, *especially Goodman's own article*, shows the need for extensive experimentation to practice the claimed method for just a few plants, let alone all plant cells as broadly claimed in the application.

Id. at 1052 (emphasis added).

Here, the disclosure of Fire, like that of Goodman, fails to provide the details necessary to practice Appellant's invention, a method of gene manipulation, in a category of hosts (mammalian cells), in the face of art-recognized doubts of the method's applicability to those hosts. The evidence of record establishes that Fire would not have enabled the skilled artisan to practice Appellant's claimed method. Not only would the amount of experimentation required to practice Appellant's claimed invention have been excessive, it would have been undue.

The first two *Wands* factors to consider are the quantity of experimentation necessary to practice the claimed invention, and the amount of guidance or direction presented. Here, the amount of experimentation would have been extensive because Fire does not contain adequate guidance. Fire does not provide any guidance regarding the practice of RNAi in human cells, for example, on how to select an amenable cell, what gene(s) can be targeted, what dose of dsRNA to use, under what conditions, for what length of time, to accomplish a measurable reduction in gene expression, while simultaneously avoiding the PKR response or without creating a generalized or nonspecific response (e.g., a "panic" response) to the added dsRNA. Fire provides only generalized recitations of cell types, ranges of potential doses, and the like. The skilled artisan cannot at once envisage what to do, or how to do it, from

Fire's generalized teachings. Experimentation would have been required to work out every detail. The required experimentation would have been undue.

The next factor to consider under *Wands* is the presence or absence of working examples. Fire provides no working examples with any vertebrate cells, nor with any mammalian cells, let alone human cells. Fire's working examples are limited to *C. elegans*. Fire exemplifies administration of dsRNA by injecting dsRNA into the gonads of larvae, feeding dsRNA to larvae, or bathing larvae in solutions containing dsRNA. Fire does not provide any working examples using isolated or cultured cells or tissues.

The *Wands* factors also include the "nature of the invention". Here, the nature of Appellant's invention is complex. The claimed invention is a method of gene manipulation in human cells. In hindsight, the methods may appear simple or even obvious to an Examiner. However, the Examiner must step into the shoes of the skilled artisan at the time of Appellant's filing. At that time, the practice of Appellant's claimed invention would have required undue experimentation.

Under *Wands*, the state of the art must also be considered. As the evidence shows, the art, at the time of Appellant's filing, did not recognize that dsRNA-induced gene silencing was attainable in *any* vertebrate system, especially mammalian systems. The presence of the known cellular defensive responses to foreign dsRNA was also of concern to the skilled artisan at the time. The prior art is full of statements doubting the existence of RNAi mechanisms in mammalian systems, and questioning the applicability of methods useful for invertebrate systems or plants to mammalian cells. The mechanisms of dsRNA-induced gene silencing were not understood in any organism, based on the knowledge in the art at the time. Significant questions remained, including, for example, what the responsible cellular systems might be, what enzymes or receptors might be involved, and what function these factors might play in the organism. Thus, the art did not know whether any such cellular systems, enzymes, or receptors were present in mammalian systems. The art failed to address or lacked any understanding of other issues such as how the various observed gene silencing systems in plants, single-celled organisms, or lower (invertebrate) animals might relate to each other, or to the more complicated systems expected in vertebrates and mammals.

Other factors to consider under *Wands* include the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims at issue. The level of skill in the arts of biotechnology and gene manipulation is high. The ordinarily skilled artisan is at least a Ph.D., possibly one with post-doctoral-level biotechnology experience.

The art of gene silencing, and RNAi in particular, at the time of Appellant's filing, demonstrated a lack of predictability with regard to success in mammalian systems. Appellant's disclosure relates to methods of RNAi in mammalian cells. The instant claims are even narrower, directed solely to methods of RNA interference in human cells *in vitro*.

In view of the foregoing, a proper analysis of the facts of this case according to the *Wands* factors shows that, at the time of Appellant's filing, the level of experimentation required to practice the claimed invention based on Fire was not only extensive, but was undue. The ultimate legal conclusion as to whether Fire would have enabled the skilled artisan, at the time, to practice Appellant's invention must be based on the factual inquiry under *Wands*. Here, Fire in combination with the knowledge in the art at the time of Appellant's filing did not put the public in possession of Appellant's claimed methods, nor allow the ordinarily skilled artisan to carry out the invention. Fire did not enable a skilled practitioner to practice Appellant's claimed invention without undue experimentation.

Appellant respectfully submits that in addition to applying an incorrect legal standard to determine whether Fire was an enabling reference, the Examiner's continued reliance on any *initial presumption* of enablement, notwithstanding the extensive evidence to the contrary, is an error. The Examiner has not adequately considered or weighed Appellant's rebuttal evidence in accordance with the requirements of the MPEP (see, e.g., *MPEP* 716.01(B)), and legal precedents set forth above.

In accordance with the foregoing, Appellant respectfully requests the anticipation rejection be reversed, and the claims be found to be novel and patentable over Fire.

II. The Claims are Not Obvious Over the Asserted References

Appellant respectfully submits that the claims are patentable over Fire in view of Kreutzer, Gewirtz, and Sharp. The rejection must be reversed because (i) the asserted combination of references does not disclose each and every element of the claimed invention; (ii) the references teach away from the claimed invention; (iii) the references and Appellant's invention were not considered as a whole, and (iv) there could have been no reasonable expectation of success at the time, given the state of the art. Further, Appellant respectfully

asserts that Kreutzer is unavailable as a reference for purposes of a rejection under 35 U.S.C. § 103 (a) having been antedated by way of a declaration by the inventor, Dr. Alan Gewirtz.

A. The Legal Standard for Obviousness Under 35 U.S.C. § 103 (a)

A claimed invention is unpatentable under 35 U.S.C. § 103 (a) if the differences between it and the prior art "are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." 35 U.S.C. § 103(a). Obviousness is a question of law, based on underlying facts. *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 17 (1966). *In re Oetiker*, 977 F.2d 1443, 1444 (Fed. Cir. 1992). Factual determinations must be supported by substantial evidence – that is "such relevant evidence as a reasonable mind might accept as adequate to a support a conclusion". See *Consolidated Edison Co. v. NRLB*, 305 U.S. 197, 229 (1938).

Recently, in *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 USPQ2d 1385 (2007), the Supreme Court considered the issue of obviousness. The Court reaffirmed that any inquiry into obviousness remains controlled by the factors set forth in *Graham.* The so-called *Graham* factors are: 1) "the scope and content of the prior art"; 2) the "differences between the prior art and the claims"; 3) "the level of ordinary skill in the pertinent art"; and 4) objective evidence of nonobviousness. *KSR*, 127 S. Ct. at 1734 (quoting *Graham*, 383 U.S. at 17-18).

In the course of prosecution in the Patent and Trademark Office, the Examiner must adhere to the following tenets of patent law to establish a *prima facie* case of obviousness:

(A) the claimed invention must be considered as a whole;

(B) the references must be considered as a whole; and

(C) the references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention;

See *MPEP* 2141 citing *Hodosh v. Block Drug Co., Inc.*, 786 F.2d 1136, 1143 n.5, 229 USPQ 182, 187 n.5 (Fed. Cir. 1986).

It is also well established, that for a *prima facie* case of obviousness there must be a reasonable expectation of success.

MPEP 2144.08 (II)(A)(1).

During examination, the examiner bears the initial burden of establishing a *prima facie* case of obviousness. *Oetiker*, 977 F.2d at 1445, *In re Piasecki*, 745 F.2d 1468, 1472, 223 USPQ 785 (Fed. Cir. 1984). The *prima facie* case is a procedural tool, and requires that the examiner initially produce evidence sufficient to support a finding of obviousness, by providing some articulated reason with some rational underpinning. *KSR*, 127 S. Ct. at 1741, 82 USPQ2d at 1396. If this initial burden is met, thereafter, the burden shifts to the applicant to come forward with evidence or argument in rebuttal. *Piasecki*, 745 F.2d at 1475. When rebuttal evidence is provided, the *prima facie* case dissolves, and the decision is made on the entirety of the evidence. *Oetiker*, 977 F.2d at 1445; *In re Spada*, 911 F.2d 705, 708 (Fed. Cir. 1990); *In re Rinehart*, 531 F.2d 1048, 1052 (CCPA 1976).

The Examiner is required to consider *all* evidence submitted in rebuttal of the *prima facie* case of obviousness of the invention. Such rebuttal evidence may include evidence relating to any of the *Graham* factors, and may include evidence of the state of the art, the level of skill in the art, and the beliefs of those skilled in the art. See *MPEP* 2144.08 (II)(B). An invention must be viewed in the context of the state of the art that existed at the time the application was filed. See *Interconnect Planning Co. v. Feil*, 774 F.2d 1132, 1138 (Fed. Cir. 1985).

An applicant may rebut a *prima facie* case of obviousness by providing a "showing of facts supporting the opposite conclusion." Such a showing dissipates the *prima facie* case and requires the examiner to "consider all of the evidence anew." *Piasecki*, 745 F.2d at 1472; *In re Rinehart*, 531 F.2d 1048, 1052 (CCPA 1976). Rebuttal evidence may show, for example, that the claimed invention achieved unexpected results relative to the prior art, *In re Geisler*, 116 F.3d 1465, 1469-70 (Fed. Cir. 1997); that the prior art teaches away from the claimed invention, *id.* at 1471; that objective evidence (e.g., commercial success) supports the conclusion that the invention would not have been obvious to a skilled artisan, *Piasecki*, 745 F.2d at 1475; or that the prior art did not enable one skilled in the art to produce the now-claimed invention, *In re Payne*, 606 F.2d 303, 314-15 (CCPA 1979).

To qualify as prior art under 35 U.S.C. § 103, a reference must also be prior art under § 102. It is also evident that, as with anticipatory references, a reference cited under § 103 must be enabling to the skilled artisan. Thus, although published subject matter is "prior art" for all that it discloses, in order to render an invention unpatentable for obviousness, the prior art must enable a person of ordinary skill to make and use the invention. *In re Kumar*, 418 F.3d 1361, 1368, 76 USPQ2d 1048 (Fed. Cir. 2005), (citing *Beckman Instruments Inc. v. LKB*

Produkter AB, 892 F.2d at 1551). Older cases, such as *Payne*, also comport with *Kumar*. *Payne* held that when a *prima facie* case of obviousness is deemed made based on similarity, for example, to a known composition or device, rebuttal may take the form of evidence that the prior art does not enable the claimed subject matter. See *Payne*, 606 F.2d at 314-15.

B. The Obviousness Rejection

Claims 1, 2, 5, 7-9, 11 and 21-38 stand rejected under 35 U.S.C. § 103 (a) over Fire in view of Kreutzer *et al.* (WO 00/44895) ("Kreutzer") (Exhibit 11, Evidence Appendix), Gewirtz *et al.* (WO 92/19252) ("Gewirtz") (Exhibit 12, Evidence Appendix), and Sharp (*Genes & Dev.* 13:139-141, 1999) (Exhibit 3, Evidence Appendix).

The Office Action mailed March 30, 2007 alleged (on page 7) that, at the time the instant invention was made, it would have been *prima facie* obvious to one of ordinary skill in the art:

> to substitute an siRNA oligonucleotide in place of the antisense oligonucleotide in a method of inhibiting the expression of the oncogene c-Kit *in vitro* using an antisense inhibitor in human leukemia cells (as taught by Gewirtz *et al.*), wherein the dsRNA would be comprised in [a] pharmaceutical composition (as taught by Fire) because antisense inhibition of c-Kit was taught in the prior art as inhibiting the expression of KitR in human leukemia cells (as taught by Gewirtz *et al.*), because dsRNA can be used to initiate RNA interference *in vitro* by targeting oncogenes in human cells including leukemia (as taught by Fire) and used *in vitro* to initiate RNA interference in mammalian cell lines (as taught by Kreutzer) and because relative to antisense approaches, dsRNA used to inhibit gene expression has advantages in the stability of the material to be delivered (as taught by Fire).

The Office Action further alleged that it would have been obvious to use a HL-60 cell line for the study of leukemia *in vitro* and further obvious to use CHP 100 to study the cellular events associated with neuroblastoma. The Office Action acknowledged that Fire does not teach the "optimal" time of incubation of said dsRNA with a cell or the "optimal" concentration of dsRNA used, but it would have been obvious to one of skill in the art and "a matter routine optimization" to determine the amount of time to expose the dsRNA to achieve the most efficient interference and to determine the optimal workable ranges of a dsRNA that most efficiently caused gene interference in a cell. The Office Action cited MPEP 2144.05 for the proposition that "where the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." (Office Action, page 8)

The Office Action further alleged that:

> [o]ne of ordinary skill in the art would have been motivated to practice a method of inhibiting the expression of the oncogene c-Kit *in vitro* in human leukemia cells or melanoma cells (as taught by Gewirtz *et al.*) using a dsRNA to initiate RNA interference wherein the dsRNA was comprised in [a] pharmaceutical composition (as taught by Fire and Kreutzer) because antisense inhibition of c-Kit was taught in the prior art as inhibiting the expression of KitR in human leukemia cells (as taught by Gewirtz *et al.)* and because relative to antisense approaches, dsRNA used to inhibit gene expression has advantages in the stability of the material to be delivered and has advantages of sequence specificity (as taught by Fire and Kreutzer).
>
> Office Action, page 8.

Finally, according to the rejection:

> [o]ne of ordinary skill in the art would have expected success in practicing a method of inhibiting the expression of the oncogene c-Kit *in vitro* in human leukemia cells (as taught by Gewirtz *et al.*) using a dsRNA to initiate RNA interference wherein the dsRNA was comprised in [a] pharmaceutical composition (as taught by Fire and Kreutzer) because antisense inhibition of c-Kit was taught in the prior art as inhibiting the expression of KitR in human leukemia cells (as taught by Gewirtz *et al.*) because Fire *et al.* teach that dsRNA can be used to initiate RNA interference in human cells and because relative to antisense approaches, dsRNA used to inhibit gene expression has advantages in the stability of the material to be delivered (as taught by Fire and Kreutzer). Moreover, one would have had a reasonable expectation of success at initiating RNA interference in human cells given that Kreutzer *et al.* successfully teach a specific embodiment of RNA interference in a mammalian murine cell line and one of ordinary skill in the art would nevertheless recognize that mice are generally representative of humans. Sharp further supports the fact that RNAi is a general mechanism that is likely to be a general mechanism for gene regulation and may be critical for many developmental and antiviral processes.
>
> Office Action, pages 8-9.

According to the Office Action, the invention as a whole would have been obvious over the asserted combination of references for those stated reasons.

C. The Asserted References

Appellant has discussed Fire at length (see discussion at section I. B., pages 6-9, *supra*) and to avoid repetition, incorporates that discussion in its entirety herein by reference, as if set forth here in full.

Kreutzer has a publication date of August 3, 2000. Appellant's filing date is November 14, 2001, with a claim to priority from a U.S. Provisional Patent Application filed November 14, 2000. Although, on its face, Kreutzer is prior art under 35 U.S.C. § 102 (a) and is properly cited in a rejection made under 35 U.S.C. § 103 (a), Appellant antedated Kreutzer during the prosecution of the instant application with the filing of a declaration by Dr. Gewirtz dated April 28, 2006 (the "Gewirtz Declaration" or "Declaration").

The Gewirtz Declaration, filed with the response to the Office Action dated December 29, 2005, establishes an effective date for Appellant prior to the August 3, 2000 date to which Kreutzer is entitled. A copy of the Gewirtz Declaration is appended hereto as Exhibit 9 in the Evidence Appendix. The current Examiner, the fourth different Examiner to process Appellant's application, failed to consider the Gewirtz Declaration. The Examiner was not the Examiner of record in April 2006 when the antedating Declaration was filed. Nevertheless, the Declaration is of record and, in view of it, Kreutzer is not available to sustain the instant rejection.

The Gewirtz reference discloses the use of antisense RNA methods for inhibiting the human c-kit proto-oncogene.

Sharp has been discussed above in response to the rejection under 35 U.S.C. § 102 (b). The discussion in section 7.1.D.3., *supra*, is incorporated herein by reference. Sharp taught that RNAi phenomena were known/established in the invertebrates, *C. elegans*, *Trypansoma brucei*, and *Drosophila*, as well as in plants. Sharp affirmatively stated that it was *not known* whether any aspect of RNAi occurs in, or can be induced in, mammalian cells.

D. The Combination of References Does Not Render the Claims Unpatentable

For purposes of this appeal, the patentability under § 103 of independent claims 1, 22, and 28, and their respective dependent claims, will be argued separately.

1. Independent Claim 1, and Claims 2, 5, 8-9, 11, and 21 Dependent Thereon are Patentable over the Asserted References

a. *The Claims*

Independent claim 1 is directed to a method for disrupting expression of a mammalian target gene at the mRNA level in a human cell. The method comprises initiating RNA

interference (RNAi) *in vitro* by exposing the human cell to a double-stranded RNA (dsRNA) homologous to the target gene. The dsRNA consists essentially of two complementary linearized strands of RNA. The transcription of each strand is independently controlled to generate paired RNAs of defined length.

b. There Would Have Been No Motivation to Combine the Asserted References

Appellant respectfully asserts that there was no motivation to combine Fire and Gewirtz. As discussed above, Kreutzer was antedated by the Gewirtz Declaration and thus not available as prior art for purposes of a rejection under § 103.

The Examiner alleged that one of ordinary skill in the art would have been motivated to combine the references because:

(i) antisense inhibition of c-kit was taught in the prior art as inhibiting expression of KitR in human leukemia cells (allegedly taught by Gewirtz), and

(ii) dsRNA used to inhibit gene expression has advantages in stability of the material to be delivered, and of sequence specificity (allegedly taught by Fire and Kreutzer). (Office Action mailed March 30, 2007, page 8). The Examiner further alleged that Sharp teaches that RNA is a general mechanism of gene regulation that may be critical for many processes.

The Examiner is incorrect. The asserted disclosure of Gewirtz is not germane to the subject matter of claims 1, 2, 5, 8-9, 11, and 21 because those claims do not have any features directed to c-kit or antisense inhibition of KitR. The Examiner has not articulated any rational basis why the skilled artisan would be motivated to combine Gewirtz with Fire **to make the invention of claim 1**. Neither the knowledge in the art at the time of Appellant's filing, nor the disclosures of Gewirtz or Fire provide any motivation to combine. There is no basis provided or reason given as to why the references should be combined, and no particular or general motivation for the skilled artisan to do so to make the invention claimed in claim 1.

The Examiner also erred to the extent Sharp is asserted as a source of motivation to combine the references. The portions of Sharp referenced by the Examiner are taken out of context. As discussed above (section 7.I.D.3., *supra*), Sharp taken as whole suggests that RNAi was an established phenomenon in invertebrates only, and that dsRNA-mediated suppression of specific genes was known in plants. Sharp concluded that it was not known whether any aspect of RNAi occurs in, or can be induced in, mammalian cells. In relying on

Sharp, the Examiner failed to consider what the reference *as a whole* would have taught or suggested to a person of ordinary skill in the art, at the time of Appellant's filing.

Finally, the Examiner must consider the state of the art as a whole, at the time of filing. At the time of Appellant's filing, the prior art did not know whether mechanisms for RNAi existed or could be leveraged for gene silencing in vertebrate, mammalian, and especially human, cells. Because the state of the art was so uncertain at the time of filing Appellant's application, the knowledge in the art itself also did not provide any motivation to combine the Fire and Gewirtz references.

c. There Would Have Been No Expectation of Success Even if the References Were Combined

Assuming *arguendo* that Fire and Gewirtz are combinable (Kreutzer is incompetent as a reference), there had to have been a reasonable expectation of success to sustain the rejection.

The Examiner alleged that one of skill would have had an expectation of success in practicing a method of inhibiting the expression of the oncogene c-Kit *in vitro* in human leukemia cells because:

(i) antisense inhibition of c-kit was taught in the prior art as inhibiting expression of KitR in human leukemia cells (allegedly taught by Gewirtz),

(ii) dsRNA can be used to initiate RNA interference in human cells (allegedly taught by Fire and Kreutzer), and

(iii) dsRNA used to inhibit gene expression has advantages in stability of the material to be delivered, and of sequence specificity (allegedly taught by Fire). (Office Action mailed March 30, 2007, page 8). The Examiner further alleged that Sharp teaches that RNA is a general mechanism of gene regulation that may be critical for many processes.

As discussed above, any contribution derived from the antedated Kreutzer reference cannot be used to sustain the rejection. The Examiner has taken Sharp out of context. In proper context, Sharp teaches that it was not known whether any aspect of RNAi occurs in, or can be induced in, mammalian cells. Given the state of the art as discussed hereinabove and throughout the prosecution, there could have been no reasonable expectation of success of arriving at the claimed invention at the time of Appellant's filing.

For example, the skilled artisan at the time of Appellant's filing did not know whether mammalian systems had the required mechanisms for RNA interference. Thus, even if the

skilled artisan were motivated to ***experiment***, success could not be reasonably ***expected*** without knowing whether the mechanisms for RNAi were (i) lacking in all mammalian cells, (ii) lacking in the selected cells, or (iii) whether a generalized response to foreign dsRNA affecting the expression of many genes was overriding silencing of a specific gene. It was not known, for example, whether an RNAi effect, if it could be induced, could be induced for ***a particular gene***, or whether only ***certain genes*** might be susceptible to silencing by RNAi. There was no basis for the skilled artisan to ***reasonably*** expect success at the time of Appellant's filing, from the combined disclosures of Fire and Gewirtz.

Only after Appellant showed in his disclosure that PKR and other cellular response mechanisms could be overcome and, thus, that mammalian cells were susceptible RNAi-induced gene-specific silencing, could there have been any expectation of success. Prior to Appellant's invention, the art lacked adequate information to allow the skilled artisan to interpret experiments, or adjust conditions to reasonably expect, or generate, a successful result.

d. The Asserted References Do Not Disclose Every Feature of the Claims

The asserted references, even in combination, do not disclose each and every feature of the claims. As above, Kreutzer was antedated by the Gewirtz Declaration. Thus, any alleged disclosure or motivation provided by Kreutzer cannot form the basis of an obviousness rejection. Fire has been discussed at length in section 7.I.B., *supra*. Gewirtz is related to antisense technology. Gewirtz discloses nothing related to RNAi, and nothing particularly related to the invention of claim 1. No claim dependent on claim 1 is currently directed to c-kit sequences described in Gewirtz.

The Examiner alleges that Fire teaches a method for inhibiting expression of a target gene using double-stranded RNA in a cell *in vitro* wherein the cell is from an animal. The rejection also alleges that Fire teaches that the cell with the target gene may be derived from or be contained in any organism and that examples of vertebrate animals include mammals and human and that the cell having the target gene may be "immortalized or transformed, or the like" (column 8, lines 52-55). The rejection also alleges that Fire states that "the present invention could be used for treatment or development of treatments for cancer of any type, including solid tumors, sarcomas, and leukemias. . ." (quoting column 10, lines 26-28).

The Office Action asserts that "the limitation 'selecting a human cell expressing the target gene' is **not *defined*** in the specification, so for prior art purposes, this recitation is

being interpreted to mean a cell line that contains a target gene and is capable of being treating [sic] with a dsRNA...". The rejection continues, alleging that Fire teaches target genes that are oncogenes in column 1, and that lipid-mediated carrier transport can be used to introduce nucleic acids to cells (citing column 9, lines 55-60 of Fire). The rejection also states that Fire teaches that inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene as determined by measurement of the target gene or expression from said target gene. Further, it is alleged (citing column 5, lines 30-37 of Fire), that Fire teaches that gene disruptions created with the methods disclosed therein, may be used to discover the function of a target gene and to produce disease models in which the target gene is involved in causing or preventing a pathological condition. Fire, according to the rejection, also discloses that relative to antisense approaches, dsRNA has advantages in the stability of the material to be delivered (citing Fire at column 3, line 20).

Contrary to the statements in the Office Action, each and every step of Appellant's claimed method is not provided in the asserted references. Appellant's claimed method expressly provides a method for disrupting expression of a mammalian target gene at the mRNA level ***in a human cell***, wherein the method comprises initiating RNA interference (RNAi) *in vitro* by exposing ***the human cell*** to a double-stranded RNA (dsRNA) homologous to the target gene, wherein the dsRNA consists essentially of two complementary linearized strands of RNA, the transcription of each is independently controlled to generate paired RNAs of defined length.

The Examiner erred as a matter of law in not giving weight to the express claim element requiring "**disrupting expression . . .*in a human cell*"**, and "**exposing *the human cell to dsRNA*.**" No authority requires Appellant to provide a definition of a claim element to be accorded weight during examination. The element is understood by the plain meaning of the words ***"in a human cell."*** The Examiner has no choice but to give full weight to this feature and is not permitted to disregard the claimed feature or to substitute a definition such as "a cell that contains a target gene and is capable of being treated with a dsRNA." The invention, i.e., the claim with all its elements, must be considered as a whole. Here, the claim expressly includes the feature **"human cell"**, which is readily understandable to the skilled artisan by its plain meaning.

Further, claim 1 requires "initiating RNAi in the human cell." Fire did not disclose, nor would the ordinarily skilled artisan be able to practice "initiating RNAi in a human cell"

or initiating RNAi in **any** mammalian cell at the time of Appellant's filing. This is evidenced by the state of the art as shown in the record and described above, e.g., as admitted by Dr. Fire and further shown by the other evidence spanning the time from Fire's filing through Appellant's filing. As discussed above with respect to the anticipation rejection, Appellant respectfully submits that Fire did not enable the skilled artisan to carry out Appellant's claimed invention, or to carry out the practice of any methods of initiating RNAi in any vertebrate cells. Accordingly, Fire did not disclose the claimed methods of *initiating RNAi in human cells*, since it did not enable the methods to be carried out. The skilled artisan having the Fire reference before him did not know how to initiate RNAi in an mammalian cell. Since Fire did not enable the practice of Appellant's claimed invention, and Gewirtz does not cure this deficiency, the combination of Fire and Gewirtz does not render the claimed invention obvious. Appellant respectfully submits that the Examiner's *prima facie* case is inadequate on its face with respect to claims 1 and claims 2, 5, 8-9, 11, and 21 dependent thereon.

e. The Prior Art Teaches Away From the Claimed Invention

The evidence of record shows that the state of the art, at the time of Appellant's filing, was fraught with uncertainty and unpredictability with respect to the application of RNAi in mammalian systems. The art teaches away from the invention of Appellant's claim 1.

Fire TiG (Exhibit 1) admitted that "the simple protocols used for invertebrate and plant systems are unlikely to be effective [in vertebrates]." Montgomery and Fire (Exhibit 2) left open their own question as to whether RNA-interference mechanisms have counterparts outside of plants and nematodes. Montgomery and Fire concluded that any gene-specific interference by dsRNA in PKR-proficient mammalian cells would require "a transient lapse in the PKR response", or an amount of dsRNA that was "incapable of activating PKR". Sharp (Exhibit 3) concluded that perhaps "some aspect of the RNAi effect occurs or can be induced in mammalian cells." After Appellant's filing date, Kreutzer (Exhibit 4) stated that despite successes in the invertebrates and plants, "until recently the general perception in the art has been that RNAi cannot be made to work in mammals." Kreutzer further stated that the prevailing belief was that "protocols used for invertebrate and plant systems would not be effective in mammals due to the interferon response, which leads to an overall block to translation and the onset of apoptosis." Finally, in 2002, also after Appellant's filing date, Paddison (Exhibit 5) professed that the ***first*** indication that the RNAi response **might** extend

to mammals came from the observation that injection of dsRNA into early mouse embryos induced sequence-specific silencing.

Because at least up until the filing of Appellant's application, the art questioned the very existence of RNAi mechanism in mammalian systems, the skilled artisan would not have looked to develop methods for practicing RNAi in mammalian cells. Skilled artisans were looking to other methods at the time. For example, as described in Fire, antisense inhibition was the most common approach during that time and substantial research effort was focused there, as well as on triple helix or co-suppression approaches. (see e.g., Fire, Background of the Invention). The art as a whole, including Fire, taught away from the invention of claim 1.

3. Independent Claim 22, and Claims 23-27 Dependent Thereon are Patentable Over the Asserted References

a. *The Claims*

Independent claim 22 is directed to a method for disrupting expression of a mammalian target gene *in vitro* in a human cell. The method comprises providing an RNA sequence homologous to a portion of the target gene, wherein the RNA is capable of inducing RNAi of the target gene. Dependent claims 23-27 include limitations wherein the target gene is c-kit and the RNA is KdsRNA in an amount effective to induce RNA interference (claim 23), or wherein the human cell resides within a population of melanoma, leukemia, tumor or transformed cells (claim 24) or the cell is malignant (claim 25). Claim 26 provides the further step of formulating the interfering RNA as part of a pharmaceutical formulation, e.g., in which the dsRNA targets a human disease or disorders in the human cell (claim 27).

b. There Would Have Been No Motivation to Combine Fire and Gewirtz

There would have been no motivation to combine Fire and Gewirtz. The motivation provided in the Office Action is as stated above:

(i) antisense inhibition of c-kit was taught in the prior art as inhibiting expression of KitR in human leukemia cells (allegedly taught by Gewirtz), and

(ii) dsRNA used to inhibit gene expression has advantages in stability of the material to be delivered, and of sequence specificity (allegedly taught by Fire and Kreutzer). Sharp

allegedly teaches that RNA is a general mechanism of gene regulation that may be critical for many processes.

Although Fire taught that double-stranded RNA is more stable than single-stranded RNA used in antisense approaches, that teaching must be considered within the context of the state of the art. As discussed above in detail, at the time of Appellant's filing, the art as a whole had substantial doubts about RNA interference in mammalian cells. It was not known whether mechanisms for RNAi existed, or whether gene-specific interference could be induced in mammalian systems. Moreover, assuming *arguendo* that there was speculation that mechanisms for RNA interference might exist, it **was** known that post-embryonic mammalian cells had overarching and generalized responses to external dsRNA that had evolved to limit the impact of foreign RNA in the cell. It **was** known in the art at the time of Appellant's filing that these generalized responses to dsRNA could lead to cell death to prevent expression of foreign RNA in a mammalian system. It ***was not*** known how to overcome these defensive responses to external dsRNA.

While Gewirtz provides the c-kit sequences, Gewirtz could not provide any motivation to resolve the issues in Fire regarding RNAi in mammalian systems. Gewirtz was solely focused on antisense technology in human cells. Accordingly, a skilled artisan at the time could not have been motivated by the combined teachings of Fire and Gewirtz, nor by any knowledge in the art at the time, to make Appellant's claimed invention. The teachings of the two references were insufficient to motivate the asserted combination. Further, there was no knowledge in the art or some other teaching that could bridge the gap in the skilled artisan's knowledge. Sharp is alleged to provide a general motivation, but considering Sharp ***as a whole***, it does not provide the missing information required by the skilled artisan to actually undertake the combination, and actually leads away from the asserted combination as it created yet more doubt as whether any such combination would be useful or successful.

Accordingly, there was no motivation for the skilled artisan to combine Fire and Gewirtz to arrive at the invention of claim 22, or claims dependent thereon, as no motivation was provided in the references themselves, nor in the art as a whole.

c. There Would Have Been No Expectation of Success; The Art Teaches Away From the Claimed Invention.

The relevant arguments concerning these factors have been set forth above for claim 1 and claims dependent thereon. Each is therefore incorporated by reference herein as an

alternative ground for reversing the rejection. Thus, even if the asserted references were combined, there could have no reasonable expectation of success in practicing the invention of independent claim 22 or dependent claims 23-27. Further, the art as whole, including Fire, teaches away from those claims, as it cast doubt on whether the mechanisms for RNAi would function for gene-specific silencing in mammalian cells, and whether the mechanisms even existed.

d. The Asserted References Do Not Disclose Every Element of the Claims

As discussed above, Kreutzer has been antedated by the Gewirtz Declaration. Thus, any alleged teachings of Kreutzer, or alleged motivation provided by Kreutzer cannot be considered in making a proper *prima facie* case.

Also, as to claims 22-27, contrary to the assertions in the Office Action, each and every step of the claimed methods are not provided in the cited references alone or in combination. Claim 22 expressly provides a method for **disrupting expression of a mammalian target gene *in vitro in a human cell***. The claimed method comprises providing an RNA sequence homologous to a portion of the target gene, **wherein the *RNA is capable of inducing RNAi of the target gene***.

Fire did not enable Appellant's invention for gene-specific silencing in vertebrate cells because the skilled artisan could not carry out those methods, as has been discussed fully above. The Examiner erred in giving no weight to the expressly claimed feature requiring **disrupting expression . . .*in a human cell***, and **exposing *the human cell***. This claim element is understood by the plain meaning of the words "*in a human cell,*" for which no definition or further explanation is required. The Examiner must give full weight to the express language of the claim, and cannot substitute a definition such as "a cell that contains a target gene and is capable of being treated with a dsRNA." Claim 22, with all its express elements and features, must be considered as a whole. Here, claim 22, as well as the dependent claims 23-27, expressly include language directed to human cells, which by its ordinary meaning is readily understandable to the skilled artisan.

Claim 22 also requires that the provided RNA be ***capable of inducing RNAi of the target gene***. Fire did not teach, or enable, the ordinarily skilled artisan to practice Appellant's claimed method. The characteristics of an ***RNA capable of inducing RNAi of a target gene*** in mammalian cells were not known prior to Appellant's filing date. This is evidenced by the state of the art, as shown in the record. As discussed above with respect to

the rejection of claim 1 for obviousness, Fire did not enable the skilled artisan to carry out Appellant's claimed invention. The practice of methods involving RNAi in vertebrate cells was not in the public's possession. Accordingly, Fire did not **teach** Appellant's claimed methods ***in human cells***, since it did not enable the methods to be carried out in human cells and did not teach any RNAs that were capable, at the time, of inducing RNAi in human cells. Gewirtz teaches treatment of human cells with antisense c-kit RNA, however Gewirtz teaches nothing about RNAi in human cells or in any cells. Fire, alone or in combination with Gewirtz, did not teach any RNA capable of inducing RNAi in a human cell. Gewirtz did not at the time provide the information lacking in Fire needed to practice the claimed invention. The asserted combination does not result in the invention of Appellant's claim 22. The skilled artisan having Fire and Gewirtz before him could not have carried out a method for disrupting expression of a mammalian target gene *in vitro* ***in a human cell*** comprising providing an RNA sequence homologous to a portion of the target gene, wherein the ***RNA is capable of inducing RNAi of the target gene.*** Appellant respectfully submits that the Examiner has not made out a *prima facie* case of obviousness with respect to claims 22-27.

4. Independent Claim 28, and Claims 7 and 29-38 Dependent Thereon, are Patentable Over the Asserted References

Independent claim 28, and claims 7 and 29-38 dependent thereon, currently stand rejected as unpatentable over the asserted references under §103, but have been deemed to be novel by the Examiner.

a. *The Claims*

Independent claim 28 is directed to a method for disrupting expression of a target gene in a human cell. The method comprises the steps of **selecting** a human cell expressing the target gene; **preparing** a double-stranded RNA (dsRNA) consisting essentially of a first strand homologous to the target gene, and a second strand complementary to the first strand; **exposing** the human cell to the dsRNA in a reaction mixture *in vitro*, under conditions permitting the dsRNA to enter the cell; and **incubating** the reaction mixture for a time sufficient to allow the initiation of RNA interference, thereby disrupting the expression of the target gene.

Dependent claim 29 details further aspects of the method including the additional steps of **measuring** the expression of the target gene in the exposed cell; and **comparing** the

expression of the target gene in the exposed cell to that of a control cell that was not exposed to the dsRNA, wherein a decrease in expression of the target gene in the exposed cell relative to that of the control is indicative of disruption of the expression of the target gene. In dependent claim 33, the measuring step encompasses measuring the amount of a protein encoded by the target gene, measuring a function of a protein encoded by the target gene, or measuring the amount of mRNA corresponding to the target gene.

In claim 30, the dsRNA has a length less than about 830 bp. Other dependent limitations include wherein the dsRNA is generated using *in vitro* transcription (claim 31), or the incubating step is about 3 days (claim 32), or the exposing step uses about 150 to 350 μg of dsRNA for each milliliter of reaction mixture. In other dependent claims, the cell is an HL-60 cell or a CHP 100 neuroepithelioma (claim 35), the target gene is c-kit (claim 36), the dsRNA is KdsRNA (claim 37), or the cell is a melanoma cell or a leukemia cell (claim 38).

b. There is No Motivation to Combine the References; There Would Have Been No Expectation of Success; and Fire and the Art as a Whole Teach Away From the Claimed Invention

As set forth above for claim 1, and claims 22-27, there would have been no motivation to combine the references, there was no reasonable expectation of success, and the skilled artisan would have been led in other directions because the art taught away from Appellant's claim 28. In particular, there is no motivation found in Fire, Gewirtz, or the knowledge in the art at the time, to combine Fire and Gewirtz. Even assuming *arguendo* that the references were combined, there was no basis on which the skilled artisan could have formed a ***reasonable*** expectation of ***success*** in arriving at Appellant's claim 28 or claims dependent thereon. The art-recognized doubts are well-documented, including in Montgomery and Fire, which is incorporated by reference into, and thus is part of Fire itself. Gewirtz adds nothing to provide the skilled artisan with a reasonable expectation of success in practicing methods of RNAi. Finally, the art, including Fire, taught away from the claimed invention because of the doubts expressed by skilled artisans that RNAi could be used for gene-specific silencing in mammalian cells, especially using the simple protocols used in invertebrate systems.

c. The Asserted References Do Not Disclose Every Limitation of the Claims

As indicated above, Kreutzer is unavailable as prior art, having been antedated by the Gewirtz Declaration. Any alleged disclosure or motivation provided by Kreutzer cannot be considered in the obviousness rejection.

The *prima facie* case fails because the asserted references, alone or in combination, do not disclose each and every element of Appellant's invention set forth in claim 28 and claims dependent thereon. Claim 28 is directed to methods for disrupting expression of a target gene ***in a human cell***. Each express claim element must be given its due weight. Here, the claim element is understood by the skilled artisan based on the plain meaning of the words used. No definition is required.

Claim 28 also requires the steps of ***selecting a human cell expressing the target gene***; preparing a double-stranded RNA (dsRNA) consisting essentially of a first strand homologous to the target gene, and a second strand complementary to the first strand; ***exposing the human cell to the dsRNA*** in a reaction mixture *in vitro*, ***under conditions permitting the dsRNA to enter the cell***; and ***incubating*** the reaction mixture ***for a time sufficient to allow the initiation of RNA interference***, thereby disrupting the expression of the target gene.

Appellant respectfully submits that the Examiner erred in not giving due weight to the express requirement for a human cell. In addition, Appellant further submits the Examiner erred in concluding that the steps of ***exposing the human cell to the dsRNA*** in a reaction mixture *in vitro*, ***under conditions permitting the dsRNA to enter the cell***, and ***incubating*** the reaction mixture ***for a time sufficient to allow the initiation of RNA interference***, are mere optimization steps.

The teachings of the asserted references alone or in combination did not enable the practice of the claimed methods in human cells at the time of Appellant's filing. This has been discussed above fully and such discussions are incorporated by reference herein.

Appellant also respectfully notes that to the extent the rejection is premised on MPEP 2144.05, it is incorrect as a matter of law. MPEP 2144.05 provides in relevant part :

> Generally, differences in concentration or temperature will not support the patentability of subject matter *encompassed* by the prior art *unless* there is evidence indicating such concentration or temperature is critical.

The subject matter of claim 28 is not *encompassed* by the asserted prior art. The Office Action acknowledges that Fires does not teach an optimal time of incubation, or an optimal concentration of dsRNA. More significantly, however, Fire does not teach ***any*** time

of incubation for exposing a human cell to dsRNA, or ***any*** concentrations of dsRNA to use for initiating RNAi in human cells.

A *prima facie* case of obviousness *may* be made when the *only* difference from the prior art is a difference in the range or value of a particular variable. *In re Peterson*, 315 F.3d 1325, 1329 (Fed. Cir. 2003); *In re Woodruff*, 919 F.2d 1575, 1578 (Fed. Cir. 1990) (emphasis added). As *In re Peterson* makes clear, an obviousness rejection under MPEP 2144.05 is, therefore, available when the *only* difference between the claimed invention and the prior art is a difference in range or value of a particular variable. Here, the combination of Fire and Gewirtz does not encompass the subject matter of claim 28 or any claim dependent thereon. The prior art failed to teach the whole genus of methods because the asserted prior art does not teach ***any*** methods that are enabled in human cells. Fire, alone or in combination with Gewirtz, does not disclose **any** range or value regarding conditions for exposing the human cells to dsRNA to allow uptake of the dsRNA, or **any** range or value regarding times for incubating the reaction mixture to allow initiating RNA interference. In short, the asserted prior art does not teach the conditions of Appellant's claimed methods. Accordingly, the Examiner has failed to present a *prima facie* case of obviousness with respect to claim 28, and claims dependent thereon.

Further, even if the claimed conditions and times were somehow found to be "encompassed" by the asserted prior art, Appellant has shown that the conditions were critical to the success of the claimed invention. As shown in the working examples, insufficient incubation time resulted in the absence of gene silencing. The working examples also showed that the RNAi response was dose-dependent. If the concentration of of dsRNA was too low, no response was seen. However, if the dose of dsRNA was too high, it was toxic to the exposed cells. Accordingly, to the extent the rejection is premised on MPEP 2144.05, it is improper, because the subject matter of claim 28 is not encompassed by the prior art, and the conditions can determine the success of the claimed invention.

III. *Conclusion*

Appellant's claims 1, 2, 5, 7-9, 11, 21-22, and 24-27 are novel over Fire and the rejection for anticipation by Fire should be reversed. The rejection relies on "vague and general language" in Fire. From the perspective of the skilled artisan at the time of Appellant's filing, Fire did not disclose Appellant's invention in an enabling fashion.

Further, the Examiner applied an improper legal standard for determining whether Fire enabled the practice of Appellant's claims.

The rejection for obviousness in view of Fire, Gewirtz, and Kreutzer should also be reversed. The Examiner failed to make a *prima facie* case of obviousness based on the asserted references. Kreutzer is not available as a reference, having been antedated. The combination of Fire and Gewirtz, would not have resulted in the claimed invention of any of independent claims 1, 22, or 28, or any claim dependent thereon. Further, the state of the art was such that there could not have been any reasonable expectation of success. The evidence of record establishes that the art lacked sufficient information to carry out the claimed methods with any expectation of success. Finally, the references themselves and the art as a whole taught away from the methods of claims 1, 22, and 28. It was widely believed, including by Dr. Fire, that RNAi mechanisms did not exist, or were not exploitable for gene-specific silencing in mammalian systems. Further, the asserted references do not teach or enable an ordinarily skilled artisan to carry out the methods claimed in claims 28-38 and claim 7 because they do not provide the critical conditions needed to practice the methods in mammalian cells, particularly human cells.

CLAIMS APPENDIX

1. A method for disrupting expression of a mammalian target gene at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro* by exposing the human cell to a double-stranded RNA (dsRNA) homologous to the target gene, wherein the dsRNA consists essentially of two complementary linearized strands of RNA, the transcription of each is independently controlled to generate paired RNAs of defined length.

2. The method of claim 1, wherein the human cell is from a cell line.

5. The method of claim 1, wherein the function of the target gene is disrupted.

7. The method of claim 28, wherein the human cell is a tumor, or a transformed cell.

8. The method of claim 7, wherein the cell is malignant.

9. The method of claim 1, comprising formulating the double stranded RNA as part of a pharmaceutical composition.

11. The method of claim 9, wherein the pharmaceutical composition comprising the dsRNA targets a human disease in the human cell.

21. The method of claim 11, wherein the human disease targeted is cancer.

22. A method for disrupting expression of a mammalian target gene *in vitro* in a human cell, wherein the method comprises providing an RNA sequence homologous to a portion of the target gene, said RNA capable of inducing RNAi of the target gene.

23. The method of claim 22, wherein the target gene is c-kit and the RNA is KdsRNA in an amount effective to induce RNA interference, thereby disrupting expression of the target gene.

24. The method of claim 22, wherein the human cell resides within a population of melanoma, leukemia, tumor or transformed cells.

25. The method of claim 24, wherein the cell is malignant.

26. The method of claim 22, comprising formulating the interfering RNA as part of a pharmaceutical formulation.

27. The method of claim 26, wherein the pharmaceutical composition comprising the dsRNA targets a human disease or disorders in the human cell.

28. A method for disrupting expression of a target gene in a human cell, the method comprising the steps of:

(a) selecting a human cell expressing the target gene;

(b) preparing a double-stranded RNA (dsRNA) consisting essentially of a first strand homologous to the target gene, and a second strand complementary to the first strand;

(c) exposing the human cell to the dsRNA in a reaction mixture *in vitro*, under conditions permitting the dsRNA to enter the cell; and

(d) incubating the reaction mixture for a time sufficient to allow the initiation of RNA interference,

thereby disrupting the expression of the target gene.

29. The method of claim 28, further comprising the additional steps of

(e) measuring the expression of the target gene in the exposed cell;

(f) comparing the expression of the target gene in the exposed cell to that of a control cell that was not exposed to the dsRNA,

wherein a decrease in expression of the target gene in the exposed cell relative to that of the control is indicative of disruption of the expression of the target gene.

30. The method of claim 28 wherein the dsRNA has a length less than about 830 bp.

31. The method of claim 30 wherein the dsRNA is generated using *in vitro* transcription.

32. The method of claim 28 wherein the incubating step is about 3 days.

33. The method of claim 29 wherein the measuring step encompasses measuring the amount of a protein encoded by the target gene, measuring a function of a protein encoded by the target gene, or measuring the amount of mRNA corresponding to the target gene.

34. The method of claim 28 wherein the exposing step uses about 150 to 350 μg of dsRNA for each milliliter of reaction mixture.

35. The method of claim 28 wherein the cell is an HL-60 cell or a CHP 100 neuroepithelioma.

36. The method of claim 28 wherein the target gene is c-kit.

37. The method of claim 36 wherein the dsRNA is KdsRNA.

38. The method of claim 7 wherein the cell is a melanoma cell or a leukemia cell.

8. EVIDENCE APPENDIX

Tab 1, Exhibit 1: "Fire TiG": "RNA-Triggered Gene Silencing." *Trends in Genetics* 15:358-363 (1999); with Abstract. Discussed in Gewirtz declaration filed September 16, 2004. Copy provided to Examiner as Exhibit 1 of Appellant's response filed January 5, 2007.

Tab 2, Exhibit 2: "Montgomery and Fire": "Double-Stranded RNA As A Mediator In Sequence-Specific Genetic Silencing And Co-Suppression." *Trends in Genetics* 14(7):255-258 (1998). Discussed in Gewirtz declaration filed September 16, 2004. Copy provided to Examiner as Exhibit 2 of Appellant's response filed January 5, 2007.

Tab 3, Exhibit 3: "Sharp": "RNAi And Double-Strand RNA." *Genes & Development* 13:139-141 (1999). Listed by the Examiner in "Notice of References Cited" on September 5, 2006.

Tab 4, Exhibit 4: "Kreutzer Patent Application": U.S. Patent Application Publication 2004/0175703, published September 9, 2004. Discussed in Appellant's responses filed April 28, 2006 at page 17, and January 5, 2007 at page 12.

Tab 5, Exhibit 5: Paddison: "Stable Suppression Of Gene Expression By RNAi In Mammalian Cells." *Proc. Nat'l Acad. Sci.* 99(3):1443-1448 (2002). Submitted as an attachment to Gewirtz declaration filed September 16, 2004.

Tab 6, Exhibit 6: Wianny: "Specific Interference With Gene Function By Double-Stranded RNA In Early Mouse Development." *Nat. Cell Biol.* 2:70-75 (2000). Listed by the Examiner in "Notice of References Cited on February 13, 2004.

Tab 7, Exhibit 7: Svodboda: "Selective Reduction Of Dormant Maternal mRNAs In Mouse Oocytes by RNA Interference." *Development (Cambridge UK)* 127:4147-4156 (2000). Listed on Information Disclosure Statement filed by Appellant April 21, 2003. Considered by Examiner on January 9, 2004.

Tab 8, Exhibit 8: . Declaration of Dr. Alan M. Gewirtz under 37 C.F.R Section 1.132, dated September 14, 2005, was submitted with the response filed September 16, 2005 to the Office Action mailed May 23, 2005.

Tab 9, Exhibit 9: "Gewirtz Declaration": Declaration of Dr. Alan M. Gewirtz For Earlier Date Of Invention Under 37 C.F.R. Section 1.131, dated April 28, 2006, was submitted with the response to the Office Action mailed December 29, 2005.

Tab 10, Exhibit 10: "Fire": Fire *et al.* (U.S. Patent No. 6,506,559), cited by the Examiner under 35 U.S.C. § 102(b) and §35 U.S.C. § 103 (a). Listed by the Examiner in "Notice of References Cited on October 6, 2004.

Tab 11, Exhibit 11: " ": Kreutzer *et al.* (WO 00/44895), cited by the Examiner under 35 U.S.C. § 103(a). Listed by the Examiner in "Notice of References Cited on December 29, 2005.

Tab 12, Exhibit 12: "Gewirtz": Gewirtz *et al.* (WO 92/19252), cited by Examiner under 35 U.S.C. § 103(a). Listed by the Examiner in "Notice of References Cited on December 29, 2005.

9. RELATED PROCEEDINGS APPENDIX

None.

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RNA-triggered gene silencing

Double-stranded RNA (dsRNA) has recently been shown to trigger sequence-specific gene silencing in a wide variety of organisms, including nematodes, plants, trypanosomes, fruit flies and planaria; meanwhile an as yet uncharacterized RNA trigger has been shown to induce DNA methylation in several different plant systems. In addition to providing a surprisingly effective set of tools to interfere selectively with gene function, these observations are spurring new inquiries to understand RNA-triggered genetic-control mechanisms and their biological roles.

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As gene-transfer technologies have become commonplace, an increasing number of organisms have been shown to exhibit potent and unexpected responses to foreign nucleic acids. The ability of some transgenes to silence the expression of homologous (chromosomal) loci was first observed in plants[1] and has subsequently been seen in nematode[2], fungal[3], insect[4] and protozoan[5] systems. Homology-dependent *trans*-silencing effects (see Box 1 for glossary) have been divided into two categories based on the nature of the effect on the target. In the first category, transcription of the target locus is unaffected, whereas the half-life of target RNAs is decreased dramatically[6,7]. Such processes have been called 'PTGS' (post-transcriptional gene silencing). A second category of homology-triggered processes exert their primary effect on the chromatin template[10], and have been termed 'TGS' (transcriptional gene silencing). A striking feature of PTGS, and of a subset of TGS phenomena, has been the existence of RNA trigger molecules responsible for the long-range effect of the transgene locus on the endogenous gene. This article will attempt to describe some emerging views, first of RNA-triggered PTGS and then of RNA-triggered TGS, while highlighting the many mechanistic questions that remain to be resolved.

BOX 1. A lexicon of gene-silencing effects

Antisense interference
Blocking the activity of genes by artificially providing complementary single-strand 'antisense' nucleic acid corresponding to the target gene.

Cosuppression
The ability of some transgenes to silence themselves and homologous chromosomal loci simultaneously.

dsRNA-triggered interference
Blocking the activity of genes by artificially providing sense and antisense RNA corresponding to a target gene.

Homology-dependent trans silencing
The ability of an RNA or DNA trigger to silence a corresponding chromosomal locus in *trans* (i.e. without any genetic linkage to the target locus).

Post-transcriptional gene silencing (PTGS)
The ability of some viruses, transgenes or RNAs to trigger the post-transcriptional degradation of homologous cellular RNAs.

Quelling
A cosuppression phenomenon in *Neurospora crassa*.

Repeat-induced gene silencing (RIGS)
(Not discussed in this paper but included in this box for clarity.) In general, RIGS refers to a localized (*cis*-acting) effect, in which regions of tandemly repeated sequence are silenced, frequently without silencing homologous genes at other sites in the genome.

RNAi
The ability to block activity of a cellular gene by injection of homologous RNA (generally used in *Caenorhabditis elegans*).

PTGS as part of a primitive immune system

In the real world, the genome of an organism must survive in a hostile environment with dangerous opportunities for unwanted gene expression and with parasites (transposons and viruses) whose interests are distinct from those of the host. This antagonism creates a need for global mechanisms that limit aberrant or unwanted gene expression. Mechanisms that are used for global genome surveillance are likely to encompass such diverse phenomena as nonsense-mediated mRNA decay[11], intron requirements for mRNA biogenesis[12], preferential methylation of transposon sequences[13] and repeat-dependent silencing[14], as well as the RNA-triggered silencing processes described in this review. In each case, a nucleic acid conformation that is not associated with normal gene expression is used by the organism as a means to recognize potentially problematic situations; in each case, one result is to block the production or expression of potentially harmful RNAs.

The best-understood biological role for an RNA-triggered silencing process comes from studies in plant systems, where a series of observations now strongly implicate PTGS as an antiviral mechanism. (1) Viral RNAs can be targets for PTGS (Ref. 1). (2) Intercellular spreading of the PTGS effect allows the plant to respond in a systemic manner after a localized viral challenge (e.g. Refs 15–17). Intracellular spreading of PTGS is thought to involve the direct dissemination of interfering RNA in a form that is reserved for genetic interference. Note that a similar spreading of the PTGS effect is also seen in *Caenorhabditis elegans* after extracellular delivery of dsRNA (Refs 18–20). (3) Several different plant viruses produce proteins that can generally interfere with PTGS (Refs 21–24). (4) Plant strains lacking PTGS are apparently healthy under laboratory conditions[21,22,25]. These strains were made using transgenic technology to produce viral anti-PTGS factors[21,22] or by direct genetic selection for lack of a PTGS response[25]. (5) Some viral infections are more harmful to plants that lack an effective PTGS response[21,22].

Although these experiments clearly implicate PTGS in an antiviral response, it is quite possible that roles will be found in control of other genomic parasites, as well as in more general aspects of cellular physiology.

Self versus non-self RNA: the nature of the PTGS trigger

Cells survive by unimpeded expression of their own genes. This implies that an RNA trigger for gene silencing must be recognized as distinct from normal cellular RNA. One potential character that might implicate RNA as foreign would be a double-stranded structure[26]; double-stranded RNA (dsRNA) is not a requisite product of normal gene expression but is produced (at least transiently) by many viruses. Strikingly, exogenous dsRNAs can act as potent triggers of PTGS in nematode[9,18], trypanosome[8], insect[27,28] and planarian[29] systems. Measurements of dsRNA potency in *C. elegans* and *Drosophila* indicate that a few molecules per cell suffice to trigger a vehement PTGS response[9,27]. Two different methods that should produce dsRNA in plants also provide reproducible triggering of PTGS: (a) the simultaneous expression of sense and antisense sequences corresponding to the desired target gene[30] (or of an RNA hairpin[30,31]) and (b) the simultaneous expression of a viral RNA replicase with a specific single-stranded RNA (ssRNA) that has been engineered to contain viral replication signals[32]. (In the latter case, note that dsRNA has been suggested but not proven as the key feature in triggering PTGS.) Because these experiments involve the deliberate introduction of aberrant RNA, they leave open several questions regarding the general nature of the PTGS trigger: (1) when and how dsRNA forms in the cell; (2) whether the formation of dsRNA is always sufficient to trigger a PTGS response; and (3) whether all PTGS involves a dsRNA trigger.

One intriguing aspect of PTGS has been the ability of transgenes that are designed to produce only sense or only antisense RNA to act as triggers[1,2,7]. Low levels of dsRNA might be produced in such cases, through the spurious transcription of both strands of a transgene, or through the transcription of inverted repeats that sometimes form when transgenes integrate[33]. As an alternative, it now seems possible that cellular RNA-dependent RNA polymerase (RdRP) could be involved in producing RNAs that can trigger PTGS. Although RdRP activity had been observed in crude extracts from several different cell types, details of the activity became clearer after an enzyme was purified and cloned from Tomato[34,35]. Genetic studies in *Neurospora* support the involvement of an RdRP in some aspect of PTGS. Screens for the loss of PTGS response to certain 'sense' transgenes led to the isolation of several mutations, one of which has been shown to result from the disruption of an RdRP-related gene[36]. Although the *Neurospora* gene product has not yet been shown to possess RdRP activity, the genetic and biochemical analysis provides an exciting link between observations in the different systems. The reaction catalyzed by the Tomato RdRP *in vitro* is a relatively non-specific conversion of

ssRNA to dsRNA. The *in vivo* specificity is likely to be highly regulated, because general copying of all cellular RNAs would wreak havoc. Analyses of the pattern of 'sense' and 'antisense' transgenes that are capable or incapable or inducing PTGS have led to several models for *in vivo* specificity for RdRP (Ref. 37). Characteristics of sense transgenes that might trigger PTGS have been proposed to include overproduction of normal RNAs, specific intramolecular secondary structure, truncations in RNA, and ineffective transcription or translation, all of which have been cited. An enticing model is that these conditions all lead, by some means (RdRP or symmetric transcription), to the production of extended regions of dsRNA.

Clearly, there needs to be tight control of any mechanism producing a trigger for PTGS. The potent response to dsRNA could pose problems for the organism, in that the accidental production of a few antisense transcripts for an important gene could provoke an unintended PTGS response. At an evolutionary level, this would be partially controlled by selection against cryptic opposite-strand promoters. Even if some accidents occurred, the combined synthesis of sense and antisense RNA in a single cell does not guarantee the formation of an interfering dsRNA. Formation of dsRNA depends on the ability of the two strands to find each other and hybridize within the dense environment of RNA-binding proteins that are present in the cell, and on the ability of the resulting duplex to resist enzymes that unwind, covalently modify, or degrade duplex RNA. Additionally, some dsRNAs might be less effective in triggering PTGS; in particular, cells might have a strong interest in triggering PTGS from foreign or aberrant dsRNA (which would signal infection) in preference to duplexes containing their own mRNA. Consistent with this hypothesis are observations in *C. elegans* (Ref. 18) and *Trypanosoma brucei* (Ref. 8), which demonstrate that triggering of PTGS by direct introduction of foreign RNA requires that both the sense and the antisense strands are provided exogenously, even if a cell already has a substantial pool of naturally synthesized sense mRNA. This is also true in plants[30], although high-level expression of sense and antisense RNAs from distinct chromosomal sites can be sufficient, in this case, to produce a PTGS response.

Can any RNA be a target of PTGS?

In each system examined, numerous mRNAs can be targets of dsRNA-triggered PTGS (Refs 8, 18–20, 27–31). Characterized targets include newly synthesized (nuclear) RNA (Ref. 9) and pre-existing cytoplasmic RNA (Ref. 8). Targeting of some nuclear RNAs argues against the direct involvement of the translational machinery, while the ability to target infecting viral RNA (Ref. 30) argues against any linkage to DNA transcription within the cell. Although sensitivity to dsRNA-triggered PTGS appears to be the rule rather than an exception, there might be some target RNAs that partially or fully resist PTGS (Ref. 20). Similarly, some tissues might be partially resistant to the effect (including some parts of the *C. elegans* nervous system; J. Fleenor and A. Fire, unpublished). Further investigation of resistant genes and cells could illuminate the study of (a) tissue- and sequence-specific aspects of the PTGS machinery, and (b) the distribution and properties of enzymes that might degrade or unwind the dsRNA trigger.

Because PTGS acts by decreasing the half-life of RNA, the natural stability of an RNA will have a quantitative influence upon its suitability as a PTGS target: naturally stable RNAs are likely to be more dramatically affected, whereas RNAs that are rapidly synthesized and degraded might be less affected. Homeostatic regulation mechanisms might also influence the final outcome of PTGS, in that a decrease in final product could activate metabolic compensation mechanisms that would partially restore expression level.

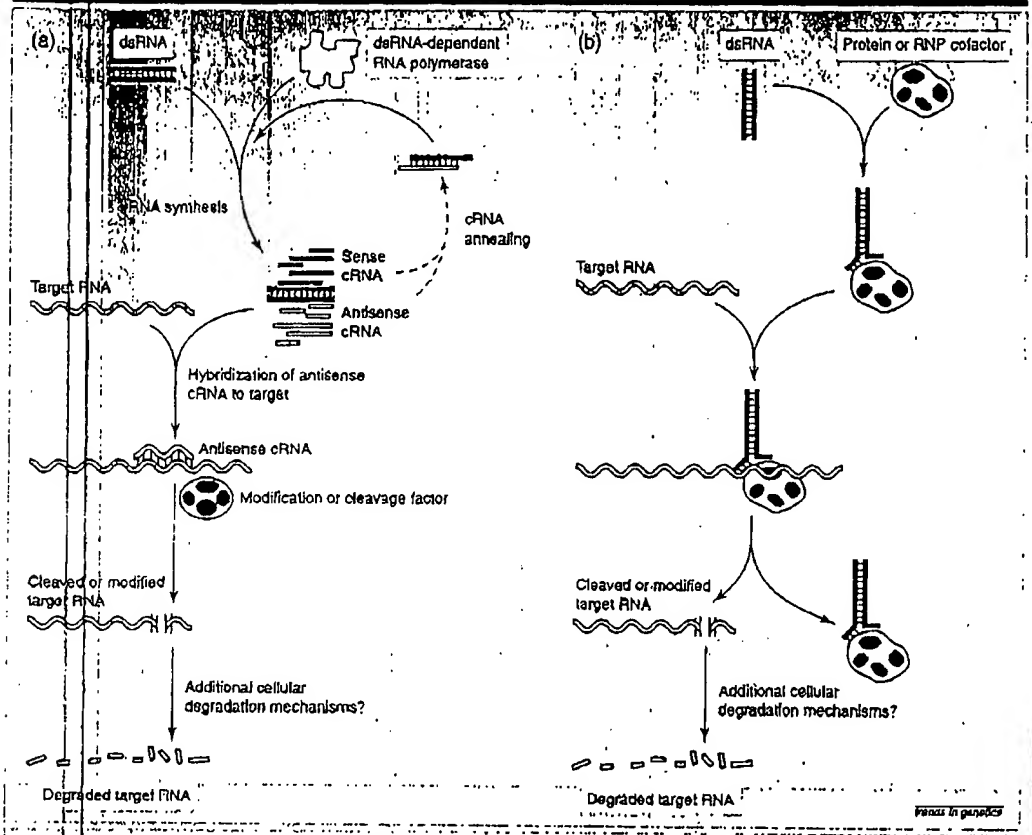
Still a mystery: mechanisms for PTGS

The ability of a few molecules of dsRNA to eliminate a much larger pool of endogenous mRNA (Refs 18, 27) suggests a catalytic or amplification component to the interference mechanism. Some of the plant literature (e.g. Refs 15, 30, 37) has favored an RNA-based copying system that is proposed to produce copious amounts of antisense RNA (while perhaps also producing additional sense and dsRNA). Direct evidence for such copy RNA (cRNA) has not been reported, although the role of an RdRP-related gene product in 'sense'-induced PTGS in *Neurospora* is certainly intriguing in this regard. Purified RdRP from Tomato is not capable of copying a dsRNA template in isolation[34]; nonetheless, it is reasonable to assume that additional enzymes could help to unwind the dsRNA product. As noted above, RdRP might alternatively (or also) contribute to PTGS at an earlier step (generating dsRNA from aberrant or unwanted ssRNA). In the absence of evidence for abundant cRNA copies of triggering RNA, an alternative to amplification is also considered. This model[9] involves the formation of a catalytic multi-round RNA-degradation machine from each dsRNA molecule. These two models (not mutually exclusive) are contrasted in Fig. 1.

To generate specificity, the initial interaction with target RNA is likely to involve hybridization, at least in a limited region. In the cRNA model of Fig. 1a, this is achieved by producing many copies of antisense RNA, which then find target RNA by hybridization. If no direct amplification is involved, then the original dsRNA must participate directly in recognition, implying a partial unwinding of the incoming RNA duplex.

After an RNA has been recognized as a PTGS target, the next challenge is to explain its decay. Recent studies of PTGS in tobacco have refined the proposal that an endonucleolytic cleavage is a key step in degradation of the target mRNA (Ref. 38). There are several ways that the initial (hybridization-based) interaction could lead to such an endonucleolytic cleavage. The least-complex model would be direct cleavage by a dsRNA-specific nuclease. Alternatively, the initial duplex might serve as a recognition site for an enzyme that then modifies the target RNA. The best candidate for such a modification would be the de-amination of adenosines to inosines, a reaction known to be catalyzed in dsRNA regions by enzymes of the ADAR family[39]. Finally, it is possible that the effect of the initial interaction with the target RNA is not a covalent change, but a positional change (sequestering the target RNA to an inhospitable region of the cell) or alteration to the spectrum of bound proteins. Whatever the initial act that modifies the target RNA, its eventual (complete) degradation is likely to require additional mechanisms. This might involve a combination of PTGS-specific degradation pathways and normal RNA decay mechanisms.

FIGURE 1. Two models for post-transcriptional gene silencing



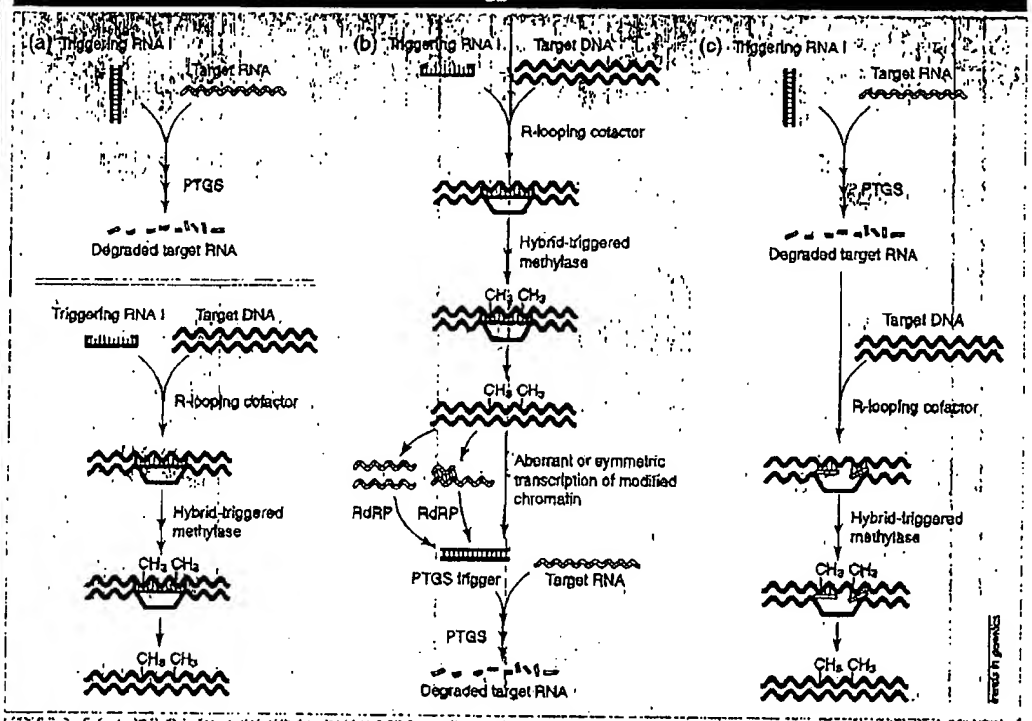
(a) A model postulating amplification of the double-stranded (dsRNA) trigger through synthesis of copy RNA (cRNA) by an RNA-dependent RNA polymerase (based on models in the plant literature; see Refs 30, 37). (b) A model that does not postulate copying once dsRNA has formed but, rather, the activity of a multi-round enzyme that can trigger the decay of many molecules of target RNA (based on Ref. 9). Unwinding of the interfering dsRNA is shown (arbitrarily) at an end of the duplex: in principal this could occur anywhere along the length of the molecule.

Is RNA-triggered chromosome modification related to PTGS?

In several plant systems it has been shown that RNA-triggered genetic silencing is accompanied by cytosine methylation (at the DNA level!) for portions of the target gene[16,40–43]. The structural identity of the endogenous or exogenous RNA that is directly responsible for triggering the modification of DNA is not known in any instance; this will be a major question of interest in the next few years. Although viral replication complexes that include dsRNA or inverted repeat transgenes are present in a number of these situations[16,40,42,43], it is conceivable that the process is completely distinct from PTGS and results from an independent interaction between the interfering RNA and the template DNA (the 'two independent process' model shown in Fig. 2a). A second model proposes one type of causal relationship, with RNA-triggered methylation of DNA causing aberrant transcription, and with the resulting transcripts inducing PTGS (Fig. 2b). Conversely, the methylation of target DNA sequences might happen as a consequence of PTGS: this model proposes that degradation products of the target mRNA (i.e. pieces of the target RNA produced by PTGS) would interact with the gene (Fig. 2c). A related model would be one in which large amounts of degraded RNA produced by PTGS might hybridize to the corresponding DNA template, causing a delay in replication that might be sufficient to induce a heritable change in the activity state of the template. If there are common components in PTGS and RNA-triggered chromosome modification, it seems likely that they will be identified by genetic analysis. In particular, it will be interesting to know the molecular identity and role of two genes in *Arabidopsis* that have effects on PTGS and on transgene-triggered DNA methylation[25].

For any model to account for sequence specificity in RNA-triggered DNA methylation, some type of RNA–DNA hybridization would be a likely component. From an experimental perspective, an observer might expect to find (1) heteroduplexes (perhaps transient) between DNA from the affected region and aberrant RNAs and (2) an enzyme that could recognize these heteroduplexes as substrates for the methylation of cytosines (or other chromosome modification, such as histone deacetylation or binding of repressive protein complexes). In cases where regions of a target gene become methylated, what is this effect on gene expression? This question adds a level of complexity, because effects on expression will depend on the exact positioning of methylated cytosines, relative to control

FIGURE 2. Possible links between PTGS and RNA-triggered chromosome modification



(a) A model postulating separate modes of action for post-transcriptional gene silencing (PTGS) and RNA-triggered chromosomal modification. An RNA of unknown structure is drawn as triggering the latter response. (b) A model in which RNA triggers DNA methylation, leading to aberrant transcription and resultant triggering of PTGS. (c) A model in which target RNA decay products from PTGS interact with the DNA template to produce a heritable change. Primary modifications to the chromosome in the diagram are arbitrarily shown as involving methylation of DNA. Alternatively, the initial modifications could involve local changes in histone modification or specific chromatin factors.

regions. The existing data from plants indicate that the heaviest RNA-triggered DNA methylation is restricted to sequences that are present in RNA (Refs 40–43). In situations where the homology between trigger and target is only within coding sequences, the methylation of the target might leave promoters and enhancers unaffected, with minimal consequent effects on expression. Under these circumstances, a strong effect would require either: (1) the presence of promoter or enhancer elements in the coding region; (2) a mechanism to nucleate a spreading of chromosomal modification from the coding region to nearby enhancers and promoters (limited spreading has been observed for viroid-induced DNA methylation in Tobacco[13]); or (3) the ability of methylation within coding sequences to impede transcriptional elongation (this has been shown for *Neurospora*)[44]. Strong effects on gene expression would be expected for triggering RNA populations that include promoter and/or enhancer sequences; this was recently demonstrated in transgenic Tobacco by Mette *et al.*[42], who showed that an active promoter could be *trans*-silenced by expression (from a second transgene) of aberrant RNAs covering the promoter. Whatever the mechanism and magnitude of the effect on gene expression, a general ability of aberrant RNAs to alter the original template could have utility in allowing a chromosome to respond to the consequences of its actions (in particular, the ongoing production of an aberrant RNA).

What is the evolutionary extent of RNA-triggered genetic silencing?

Given the diverse phylogenetic positions of plants, nematodes, fungi, flies, planaria and trypanosomes, it seems certain that the distribution of RNA-triggered genetic silencing in the biosphere is extensive. Examples are likely to accumulate as gene transfer (or RNA injection) is attempted in additional organisms. The initial observation will often be *trans*-silencing by foreign or engineered DNA. Frequently, the lack of a promoter in the incoming DNA (or failure to find RNA transcripts) is taken as evidence for lack of an RNA intermediate: this type of evidence should be interpreted cautiously, because transgenes are frequently transcribed at low levels on both strands and a few molecules of dsRNA would rarely be detected in these experiments. Some *trans*-acting genetic effects are definitively not triggered by RNA (e.g. transvection effects in *Drosophila* triggered by DNA–DNA pairing[45]). Nonetheless, it seems prudent to carefully consider a possible RNA trigger (particularly dsRNA) as each new homology-dependent *trans*-silencing phenomenon is characterized.

Real-world applications: what about us?

Procedures based on RNA-triggered silencing are now well-established tools for functional genomics of lower organisms (plants, invertebrates and fungi). Valuable information about gene function can be obtained, even in

cases where only a partial loss-of-function is generated. From a technical perspective, one could certainly hope that RNA-triggered silencing would exist in vertebrates: this would facilitate functional genomics and might allow medical applications involving targeted silencing of 'renegade' genes. Although this hope is not ruled out by any current data, the simple protocols used for invertebrate and plant systems are unlikely to be effective. Mammals have a vehement response to dsRNA, the best-characterized component of which is a protein kinase (PKR) that responds to dsRNA by phosphorylating (and inactivating) translation factor EIF2a (Ref. 46). As with nematode and plant systems, mammalian cells can respond to extracellular dsRNA and, thus, might have a specific transport mechanism to bring the dsRNA to the intracellular PKR enzyme. Controlled-delivery studies suggest that a single molecule of dsRNA within the cell can trigger an overall cellular response[47]. Any gene-specific dsRNA response in mammals would need to exist in cells or conditions where PKR is less effective, or would need to work in the shadow of the PKR-induced global response. Nonetheless, a recent report of co-suppression in mammalian cells[48], and the implication of RNA triggers with a potentially double-stranded character in a number of natural genetic interference processes (X-inactivation[49] and imprinting[50]) suggest the possibility that some components of RNA-triggered silencing machinery could be conserved from lower organisms.

Even if the underlying mechanisms are absent in mammals, it is possible that RNA-triggered silencing will have clinical applications. In particular, the ability to silence essential parasite genes (thereby limiting a parasitic infection) could be of great value. Of course, the dsRNA would have to be delivered so as to avoid harming the host. The PKR system (although non-essential for survival in mouse models[46]) is sufficiently ubiquitous that interfering with it might be counterproductive. An alternative would be to find chemical modifications to the dsRNA that would still enable it to function in gene-specific interference (e.g. in a parasite), while not inducing the PKR response in the host.

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ELSEVIER SCIENCE FULL-TEXT ARTICLE

RNA-triggered gene silencing.

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Double-stranded RNA (dsRNA) has recently been shown to trigger sequence-specific gene silencing in a wide variety of organisms, including nematodes, plants, trypanosomes, fruit flies and planaria; meanwhile an as yet uncharacterized RNA trigger has been shown to induce DNA methylation in several different plant systems. In addition to providing a surprisingly effective set of tools to interfere selectively with gene function, these observations are spurring new inquiries to understand RNA-triggered genetic-control mechanisms and their biological roles.

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into an exon or intron of an actively transcribed endogenous gene, thereby simultaneously disrupting the gene and acting as a locus-specific marker of the gene. This would greatly facilitate the identification and cloning of the disrupted genes, a task that is not necessarily straightforward following chemical mutagenesis. In practice, hundreds of transgenic embryos, each carrying multiple integration sites, could be generated per day and screened for GFP expression. A huge advantage of using this approach in frog embryos is that they develop externally, therefore GFP expression can be assayed in living embryos at any stage. Most embryos will not express the marker gene. These will be discarded and only the few that express will be nurtured to maturity, thus greatly reducing the number of embryos that must be carried to the next generation. Preliminary experiments in *X. laevis* strongly suggest that using a gene trap approach will be productive (O. Bronchain and E. Amaya, unpublished).

The powerful manipulations that one can perform on amphibian embryos have been used to reveal important principles about development for over a century. As we approach the next century, it appears that it will now be possible to overlay this rich embryological history with the power of genetic manipulations, creating an armamentarium of approaches as we look towards revealing a new generation of concepts about vertebrate embryonic development.

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Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression

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Many fundamental natural processes have been uncovered not by preplanned scientific enquiry, but serendipitously by engineers and scientists who observed unexpected consequences of their manipulations. Biologists routinely use engineering to manipulate the expression of specific genes and, thus, understand (or benefit from) their function. Sometimes we wish to make a particular gene silent; at other times we want the genes to 'talk' more loudly. Attempts at silencing have often employed an antisense strategy of introducing single-stranded nucleic acid from the noncoding strand to sequester or modify the native transcript, thereby preventing accumulation of the corresponding protein. Conversely, by introducing extra copies of a specific gene, one might expect in many cases to overproduce the corresponding mRNA and protein products. Although these techniques have been successful in numerous applications, a body of literature is emerging that documents certain cases in which unexpected outcomes of these manipulations are seen in organisms as diverse as nematodes and plants. These observations encompass 'transgene silencing' (a failure to express certain multicopy transgenes) and co-suppression (the ability of a 'sense' transgene to interfere with the activity of the endogenous genetic locus). Certain of these phenomena are thought to involve direct DNA–DNA interactions, whereas others have been proposed to require an RNA effector molecule. The structure and mechanistic properties of RNAs mediating the latter type of co-suppression have yet to be elucidated. Here, we discuss the possibility that double-stranded RNA (dsRNA), rather than sense or antisense single-stranded RNAs alone, is the effector molecule responsible for RNA-mediated silencing and co-suppression.

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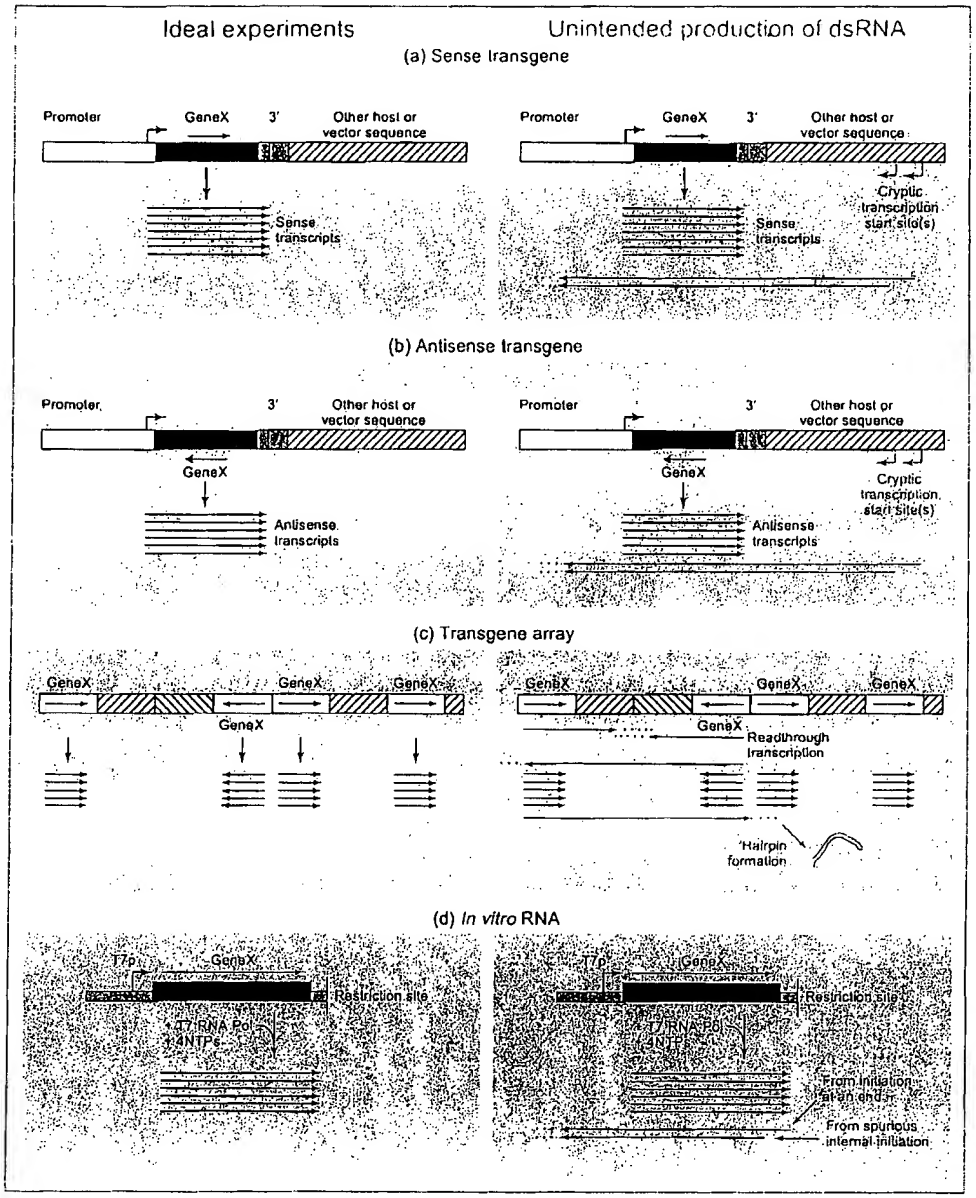

FIGURE 1. Unintended production of double-stranded (ds) RNA. Left, a series of theoretical experiments that might be designed to produce a pure population of single-stranded RNA. Right, how a low level of dsRNA could also be produced in each case. (a) A transgene designed to produce 'sense' RNA is transcribed at low level from a cryptic (or natural) start site on the opposite strand. Hybridization to 'sense' transcripts from the same template would result in dsRNA. (b) A transgene designed to produce 'antisense' could similarly be subject to low-level transcription on the opposite strand, with dsRNA resulting from hybridization of the newly transcribed RNAs. (Note that antisense RNA might alternatively hybridize with the endogenous chromosomal transcript to make dsRNA; it is not clear, however, that sense and antisense RNAs sythesized at distant nuclear sites would form dsRNA and be capable of interference.) (c) A transgene array containing tandem and inverted copies of a DNA construct ('geneX') might be expected to produce only one strand of RNA. Note, however, that readthrough of the geneX terminator would produce RNA with an inverted repeat structure. This RNA could undergo intramolecular hybridization to produce a predominantly double-stranded hairpin. (d) During *in vitro* synthesis of RNA, transcription initiates primarily at the bacteriophage RNA-polymerase promoter. Initiation can, however, also occur at internal sites and template ends, which leads to some inclusion of dsRNA in 'sense' and 'antisense' RNA preparations.

RNA-mediated genetic interference (RNAi) in the nematode *C. elegans*

Several years ago it was reported that antisense RNA targeted to specific endogenous genes in *Caenorhabditis elegans*, when either expressed from a transgene[1] or injected directly into the worm's gonad[2], could phenocopy a null or hypomorphic mutation in the targeted gene. Surprisingly, both reports indicated that sense transcripts also were effective in producing the targeted phenotype. These observations were difficult to explain using a conventional model of antisense sequences inactivating the sense transcript. Recently, at least part of this mystery was solved by the discovery that much, if not all, of the genetic interference from injected 'sense' and 'antisense' RNA is actually mediated by double-stranded RNA (dsRNA) that is present at a low level in all *in vitro* RNA syntheses because of the non-specific activity of RNA polymerases[3] (Fig. 1). Highly purified preparations of antisense (and sense) RNAs had negligible effects, whereas dsRNA exhibited potent and specific interference against any of a variety of targeted genes. The potency of RNAi in worms points to the existence of novel mechanism(s)[3]. Even with an abundantly transcribed target (several thousand mRNA copies per cell), a few molecules of dsRNA per cell can produce specific inhibition. This would not be expected from a simple antisense mechanism; there is simply not enough material to bind to all the endogenous mRNA targeted for destruction. These results generated two fundamental questions: how can dsRNA mediate gene-specific interference; and what is the physiological purpose of this process?

RNA-mediated silencing and co-suppression in plants

A second body of work on RNA-mediated interference comes from the plant world[4]. In the late 1980s, plant researchers were surprised to find that the introduction of certain transgenes into plants can result in homology-dependent silencing of an endogenous locus (rather than overexpression of the coding region of interest). This phenomenon is referred to as co-suppression. Not all transgenes cause this effect; there is no current basis for predicting which would and which would not. Gene silencing in plants has been proposed to encompass a variety of different mechanisms[4,5], including some that act by direct DNA–DNA interaction and others that involve interference by an RNA product of the transgene. Strong evidence for the latter class of mechanisms comes from experiments in which RNAs are introduced in the absence of a DNA template (using RNA viruses as vectors); the ability of viral RNAs to interfere with a homologous gene in the plant genome is one of the strongest arguments for the existence of RNA-mediated silencing mechanisms[6–8].

The literature contains a few clues as to the nature of the interfering RNA. In certain cases, co-suppression is correlated with high-level transcription of the transgene[9,10]. Given recent results in *C. elegans*, we pose the possibility that transcription along the antisense strand of a transgene could result in low levels of interfering dsRNA (Fig. 1). Such transcription might be low-level synthesis directed by sequences within the vector or flanking regions at the site of integration (e.g. see Ref. 11). Significantly, Que *et al.*[10] reported that, whereas co-suppression was associated with accumulation of transcripts at high concentrations from single-copy transgenes, inversely repeated transgenes could cause co-suppression, irrespective of promoter strength or level of the transgene mRNA. Transcripts from inversely repeated transgenes would be expected to produce a 'double-stranded structure. Experiments with chimeric RNA viruses[7,8] might similarly point toward a dsRNA involvement; in these experiments, the viral RNA replicase copies the chimeric RNA in the cytoplasm, generating both sense and antisense material.

Similarities between nematodes and plants

RNAi in worms and co-suppression in plants share some striking similarities. Both are cases of gene-specific interference. dsRNA has been shown to be the agent of interference in nematodes and, as suggested above, there is some indication that dsRNA could also be responsible for co-suppression in plants. Perhaps the most interesting common characteristic is that the phenomenon can spread from the site of interfering RNA synthesis or application. In worms, the dsRNA mix can be injected into the body cavity, where it can produce an interfering effect in distant tissues and in F1 progeny, indicating that cells may have an RNA-transport mechanism[3]. Similarly, two groups of researchers have demonstrated the systemic spread of co-suppression in plants[12,13]. An RNA molecule, spreading throughout the plant via phloem, has been proposed as the mobile agent responsible for transmitting the co-suppression state[12].

Possible mechanisms for RNA-mediated interference

The sub-stoichiometric activity of the interfering RNA in *C. elegans* led to various models: that interference involves a catalytic mechanism dependent on the injected RNA; that the input material is amplified; or that interference occurs at the level of the gene. Several lines of evidence argue against DNA in the genome as a target for RNAi. Effects of dsRNA are generally not heritable beyond the first generation; injected animals and progeny exhibit the effects of RNAi, whereas animals of the F2 generation generally revert to a wildtype phenotype[3]. Additional evidence comes from direct sequencing of genomic DNA following RNA-mediated interference with *unc-22*; these experiments yielded no indication of mutations in the target gene (S. Xu and A. Fire, unpublished). Consistent with an RNA target, interference was effective using a variety of regions present in mature RNA, but was not effective using intronic or promoter sequences[3]. At this point, one attractive hypothesis is that dsRNA might result in early degradation of the endogenous mRNA. We know from *in situ* hybridization studies that RNA transcripts of a target gene fail to accumulate after RNAi (Ref. 3). Conceivably, the lack of mRNA products could be an indirect consequence of blocked processing or transport. Alternatively, endogenous transcripts could be degraded by a sequence-specific mechanism directed by dsRNA.

The mechanisms mediating certain co-suppression phenomena in plants have been shown to act both on the DNA template and on RNA products. Wassenegger *et al.*[6] showed that viral or transgene-generated RNA could direct *de novo* modification (presumably methylation) of a homologous sequence in the plant genome. Other studies provide cases in which RNA-mediated co-suppression acts

post-transcriptionally, potentially by the rapid degradation of the target transcript[14]. In one example, transgene-mediated silencing of the endogenous gene encoding β-1,3-glucanase in tobacco, de Carvalho Niebel and colleagues[15] demonstrated that the suppressed genes are actively transcribed. Subsequently, Jacobs *et al.*[16] showed that gene silencing in this line correlates with an increased turnover of both the transgenic and the endogenous transcripts of β-1,3-glucanase. In the case of virally provided RNA sequences, it appears that viral RNA molecules can serve as targets as well as 'triggers' for co-suppression[8,13,17]. Could there be a mechanistic link between (a) RNA-mediated degradation of RNA and (b) RNA-mediated methylation of DNA? One possibility is that these are two separate processes mediated by similar RNA molecules; alternatively, there could be a causal relationship, perhaps from an ability of RNA decay products to trigger methylation of homologous sequences in replicating DNA.

Does RNA-mediated interference do a job for the cell?

In addition to the mechanistic questions, attention is also merited to the physiological role for the RNA-associated silencing phenomenon. A role for co-suppression mechanisms in systemic defense against viruses has been suggested for plants[17,18] and could apply to other organisms as well. Such a response represents an effective means by which to prevent viral replication and induce resistance in surrounding tissues prior to viral invasion.

Alternatively, co-suppression/RNAi might modulate normal gene expression. One can easily imagine double-stranded RNAs being used by the cell as a potent means to turn off specific genes in response to physiological or developmental cues. Perhaps the best way to identify these processes will be to find mutants that are defective in carrying out RNAi.

Do RNA-interference mechanisms have counterparts outside of plants and nematodes?

Mammalian cells exhibit a global antiviral response to double-stranded RNA. In this response, the PKR protein kinase recognizes dsRNA and unleashes a vehement but somewhat non-specific response leading to general translational arrest[19]. Intriguingly, this type of systemic response can occur if the dsRNA is provided extracellularly[20] (consistent with the possibility of dsRNA uptake by mammalian cells). Viruses have evolved a number of strategies for evading or inhibiting the PKR response[21]. Certain tissue-culture cell lines lack PKR and are susceptible to mutant viruses that would otherwise be non-virulent. Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of activating PKR.

A wealth of information indicates that specific RNA-mediated interference mechanisms contribute to the control of gene expression in vertebrate and other systems. For many of these contributions, the precise nature of the interfering RNA (single-stranded versus double-stranded material) has yet to be characterized. Antisense transcripts have been reported for large numbers of vertebrate genes[22]. In some cases, roles for these transcripts in regulating the sense transcripts from the opposite strand have been demonstrated. From an informatics perspective, a surprisingly large fraction of vertebrate mRNAs contain long-conserved sequences within the 3′ untranslated region as well as long blocks without silent changes in their protein-coding regions[22]. Lipman[22] has proposed that these conserved sites are regulatory targets of endogenous antisense transcripts encoded by the complementary strand of the gene. Such a mechanism would, thus, be common and relatively conserved. Endogenous genes regulated by antisense transcripts have also been described for the primitive eukaryote *Dictyostelium*, and such mechanisms have been studied in detail in Eubacteria and Archaebacteria (reviewed in Ref. 23). Co-suppression phenomena, similar to that described for plants, have also been observed in *Dictyostelium*[24]. It will be interesting in the next few years to learn whether any or all of these effects share underlying mechanistic features and we suggest, moreover, that by studying the mechanisms underlying these phenomena, we will be better able to interpret the native language of the cell.

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PERSPECTIVE

RNAi and double-strand RNA

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Double-strand RNA (dsRNA) is a signal for gene-specific silencing of expression in a number of organisms. This phenomenon was demonstrated recently in *Caenorhabditis elegans* when dsRNA was injected into the worm and the corresponding gene products disappeared from both the somatic cells of the organism as well as in its F_1 progeny (Fire et al. 1998). This RNA interference, RNAi, has been generalized to many genes in *C. elegans* (Montgomery and Fire 1998; Shi and Mello 1998; Tabara et al. 1998; Timmons and Fire 1998). ds-RNA can also suppress expression of specific genes in plants, a component of the phenomenon called cosuppression (Vionnet et al. 1998; Waterhouse et al. 1998). Two recent reports document dsRNA-mediated interference with expression of specific genes in other organisms. Double-strand RNA produced gene-specific phenotypes in *Trypanosoma brucei* (Ngo et al. 1998) and, very recently, dsRNA-mediated interference was demonstrated in *Drosophila* (Kennerdell and Carthew 1998). Thus, the RNAi phenomenon is likely to be a general mechanism for gene regulation and may be critical for many developmental and antiviral processes.

Montgomery et al. (1998) have investigated how RNAi suppresses the expression of endogenous genes in *C. elegans*. dsRNA might conceivably direct mutations of the endogenous genes thus inactivating function. However, the fact that the F_2 progeny from RNAi-treated *C. elegans* generally reverted to normal phenotype argued against nonreversible gene modification. Further sequencing of the targeted locus failed to detect nucleotide differences, direct evidence against a mutational mechanism.

Double-strand RNA primarily suppresses gene expression by a post-transcriptional mechanism in *C. elegans* (Montgomery et al. 1998). A post-transcriptional mechanism was foreshadowed by earlier experiments showing that dsRNAs from sequences in the mature RNA, that is, exons, had RNAi activity, whereas dsRNAs from intron sequences did not (Fire et al. 1998). The most direct evidence for a post-transcriptional effect arises from analysis of RNAi effects on a multiple-gene operon. Such operons are expressed in *C. elegans* by transcription of long precursor RNAs that are then processed by trans-splicing and cleavage to generate specific mRNAs. The *lin-15b* and *lin-15a* genes are part of one operon and both need to be inactivated to generate the multivulva phenotype. Injection of dsRNA from both genes generated the phenotype, whereas injection of dsRNA from either gene alone did not. This strongly indicates that suppression of the upstream gene does not inactivate the downstream gene and thus that the RNAi effect is post-transcriptional. The post-transcriptional effects of RNAi were directly observed using in situ hybridization to follow transcripts of genes suppressed by injection of dsRNA (Montgomery et al. 1998). There was a diminution of nuclear RNA from the suppressed gene as well as a total absence of the specific mRNA in the cytoplasm. This suggests that dsRNA establishes an intracellular state that destroys RNA transcribed and spliced from a specific gene. Both this study and other results are most easily explained if the specific RNA degradation occurs in both the nucleus and the cytoplasm.

dsRNA mediated suppression of specific gene expression has also been observed in plants. One demonstration of the phenomenon follows expression in plant cells of a recombinant RNA virus containing exonic sequences of an endogenous cellular gene. Expression of the cellular gene is suppressed in these cells when the recombinant viral RNAs are capable of replicating and not when they are replication incompetent (Angell and Baulcombe 1997). Viral RNA replication involves dsRNA. A similar phenomenon can be observed when a transgene is introduced into plant cells. The endogenous gene corresponding to the transgene can become suppressed (e.g., Vionnet et al. 1998), perhaps due to symmetric transcription of both strands of the transgene. Such symmetric transcription could arise by initiation in flanking sequences due to the presence of fortuitous promoter sites in plasmid DNA. The plant and nematode effects share the property of spreading. Examples of this are striking. Worms fed dsRNA exhibit a strong systemic interference phenotype (Timmons and Fire 1998) and introduction (into plants) of 500-bp fragments of a gene absorbed on the surface of a gold bead projectile can result in suppression of the gene in cells both immediately adjacent to the site penetrated by the bead as well as at very distant sites (Vionnet et al. 1998).

The purest demonstration that dsRNA mediates gene silencing in plants is the genetic study of Waterhouse et al. (1998). Transgenic plants were established which expressed either sense or antisense of a gene of the potato

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virus Y (PVY). Both transgenic lines of tobacco were susceptible to PVY infection. However, crosses of these tobacco lines that expressed transgenes for both the sense and antisense orientation and thus could generate dsRNA became resistant to PVY. This suggests that the two complementary RNAs transcribed from unlinked loci were able to anneal in the nucleus and induce a gene-specific suppressive state.

There are indications in *C. elegans* and direct evidence in plants that dsRNA may also act by generating a second mechanism, a silencing of transcription of a specific gene. In plants, the silenced gene has been shown to be hypermethylated, perhaps contributing to the inactive state (Wassenegger et al. 1994). In *C. elegans*, the only indication of such a gene-linked suppression is that the RNAi effect for some genes can be transmitted to the F_2 generation (Tabara et al. 1998). Silencing of an endogenous locus by transgenes has also been observed in *Drosophila* (see, e.g., Pal-Bhadra et al. 1997). In these cases, the copy number of the transgene appears to be important and there is no direct evidence that the silencing mechanism is mediated by dsRNA. One study strongly suggests that the mechanism of silencing is transcriptional, with the proteins of the polycomb complex becoming associated with the silenced endogenous locus (Pal-Bhadra et al. 1997). Polycomb complexes are known to be important for the silencing of genes during development. In the study, where dsRNA has been shown to be a potent and specific inhibitor of gene activity in *Drosophila* (Kennerdell and Carthew 1998), transmission through the germ line was not observed. In this case, the dsRNA was injected into the syncytial blastoderm embryos and it generated phenotypes in the L1 larvae but not in the progeny.

The finding that dsRNA may induce the transcriptional silencing of a specific gene could be important in several biological phenomena. For example, recent results suggest that the activity of an antisense promoter in the first intron of the gene for the mouse receptor for the insulin-like growth factor type-2 (Igf2r) is important for its paternal-specific repression (Wutz et al. 1997). In this case, dsRNA generated by symmetric transcription from the two opposing promoters might be a signal for establishing the allele-specific repression.

The cosuppression phenomenon and particularly its post-transcriptional gene silencing aspect (PTGS) in plants has been studied for a number of years. Several startling conclusions from these experiments may have implications for the RNAi effect in other organisms. First, that the PTGS effect, whether induced by a DNA segment or by a RNA duplex as part of the replicative intermediates of a RNA virus, is probably present in the cytoplasm as it inhibits specific gene expression from RNA viruses that are not known to enter the nucleus. Second, the gene-specific agents that stimulate or mediate the cosuppression effects are amplifiable by normal cells and will spread through plants by both plasmodesmatal and phloem channels (Vionnet et al. 1998). Furthermore, the agent(s) of suppression will pass through cells that do not contain any endogenous gene affected by the cosuppression effect. This suggests that the agent, which is likely to be composed of nucleic acids because of its gene-specific origin and effect, can be transmitted, and probably amplified, by cells utilizing the genetic information in the agent alone.

Evidence for amplification of the gene-specific agent comes from comparison of the amount of input dsRNA and the number of cells and number of mRNAs in those cells which are suppressed. For example, plant tissue with the PTGS state can be grafted onto other normal plants with a resulting spread of the PTGS state into the tissue of the grafted host (Vionnet et al. 1998). Similarly, a limited amount of dsRNA will generate RNAi in both the somatic tissue of the worm and the F_1 progeny (Fire et al. 1998). The only alternative explanation for the remarkable efficiency of the RNAi other than amplification would be the establishment of a highly catalytic RNA degradation process. It is possible, in fact likely, that both an amplification process as well as a catalytic process are part of the RNAi phenonemon in some organisms.

Restriction of infection by RNA viruses is almost certainly one of the biological consequences of the PTGS or RNAi state. Not surprisingly, some plant viruses appear to encode gene products which block the development of the PTGS state (Kasschau and Carrington 1998). The mechanism of suppression of the dsRNA induced gene-specific silencing by the viral protein remains to be investigated.

The implications of a dsRNA-induced gene-specific silencing mechanism that is amplified by normal cells are astounding when considered in molecular terms. The effects discussed above can be diagrammed as follows:

dsRNA containing exons of gene X
↓
RNAi or PTGS
↓
agent causes gene-specific degradation of mRNA from gene *X* in both nucleus and cytoplasm; gene-specific agent, or inducing agent, can be duplicated by a normal cell and transmitted to other cells.

Speculation about the molecular processes, which underlie RNAi effects, might begin with a previously described mechanism for covalent modification of dsRNA (Bass and Weintraub 1988). Most eukaryotic cells contain one or more adenosine deaminases which convert many of the A residues in duplex RNA to inosine (I). The product RNA becomes sufficiently modified that the two strands of the RNA dissociate. This I-containing RNA is bound by proteins in extracts, so far not characterized, that are stable to electrophoresis in native complexes (Wagner and Nishikura 1988). One can speculate that this modified single strand RNA with bound proteins could be the agent, which interacts with nuclear and cytoplasmic RNAs, signaling their degradation. This agent might also be replicated by cellular polymerases, perhaps RNA polymerase II, which is thought to repli-

cate viroid-type RNAs in both plant and vertebrate cells. If replicated by a base-pairing mechanism, the I residues would be converted to G residues in the product. This type of conversion has been documented during the replication of RNA viruses where it is manifested as hypermutation of the viral genome (Bass 1997). Adenosine deamination of nuclear RNA has been characterized for sense and antisense RNAs from the early region of the DNA virus polyoma (Kumar and Carmichael 1997). In early-strand RNA recovered from late infected cells, approximately half of the adenosines were modified. Interestingly, this modified RNA was primarily confined to the nucleus and was apparently relatively stable.

In vertebrate systems, dsRNA was long ago recognized as a potent signaling molecule in the induction of interferons and execution of the antiviral state. Briefly, exposure of cells to dsRNA, such as poly(IC), potently induces the transcription of interferons that induce an antiviral state in cells. The antiviral state is characterized by the synthesis of a number of proteins that recognize dsRNA, a common property of the replication intermediates of RNA viruses. These proteins include a kinase, PKR which is activated by dsRNA, a 2′–5′-oligoadenylated synthetase activated by dsRNA and dsRNA-specific adenosine deaminase activities. The PKR kinase activity suppresses translation by phosphorylation of initiation factors and synthesis of oligo-2′–5′ poly activates the endoribonuclease RNase L, which degrades RNA. However, neither this suppression of translation nor degradation of mRNA has been shown to be gene-specific in action. In fact, it remains a mystery how cells treated with interferon specifically suppress the translation of viral mRNAs in their cytoplasm and not cellular mRNAs. Perhaps some aspect of the RNAi effect occurs or can be induced in mammalian cells.

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Stable suppression of gene expression by RNAi in mammalian cells

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In a diverse group of organisms including plants, *Caenorhabditis elegans*, *Drosophila*, and trypanosomes, double-stranded RNA (dsRNA) is a potent trigger of gene silencing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function. Use of RNA interference (RNAi) as a genetic tool has recently been extended to mammalian cells, being inducible by treatment with small, ~22-nt RNAs that mimic those produced in the first step of the silencing process. Here, we show that some cultured murine cells specifically silence gene expression upon treatment with long dsRNAs (~500 nt). This response shows hallmarks of conventional RNAi including silencing at the posttranscriptional level and the endogenous production of ~22-nt small RNAs. Furthermore, enforced expression of long, hairpin dsRNAs induced stable gene silencing. The ability to create stable "knock-down" cell lines expands the utility of RNAi in mammalian cells by enabling examination of phenotypes that develop over long time periods and lays the groundwork for by using RNAi in phenotype-based, forward genetic selections.

The use of genetically tractable model systems has been the key to our present understanding of gene structure and function, cell and organismal biology, and, ultimately, the molecular aspects of human disease. The ability to stably knock out or knock down gene expression and, thus, function, in particular, has been paramount to the use of such models for illuminating biological function. For example, the use of conditional lethals in bacteriophage T4 allowed functional analysis of phage morphogenesis modules (1), whereas the same technique applied to yeast permitted the discovery of functional hierarchies among genes regulating cell cycle progression (2, 3). In both scenarios, cells acquire stable phenotypes through heritable genetic alterations.

Although such basic genetic approaches are virtually effortless in many model organisms, cultured mammalian cells have proven somewhat intractable, in this regard. This is largely because cultured mammalian cells are diploid and favor nonhomologous over homologous recombination. Current approaches to create stable phenotypes in mammalian cells have been often met with limited success. Dominant-negative and antisense strategies have proven inconsistent and unpredictable, thus lacking experimental rigor equivalent to a point mutation in yeast. However, one approach now used extensively in other diploid organisms has the potential to foment a revolution in mammalian somatic cell genetics. This approach is dubbed double-stranded RNA (dsRNA)–dependent posttranscriptional gene silencing, or RNA interference (RNAi).

It has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans (reviewed in ref. 4). A combination of genetic and biochemical studies suggests that many of these phenomena share a common mechanism. The prevailing model begins with the conversion of the dsRNA silencing "trigger" into small RNAs (guide RNAs or siRNAs, ref. 5) that range in size from ~21 to 25 nts, depending on the species of origin (6–8). These RNAs become incorporated into a multicomponent nuclease complex, which uses the sequence of the guide/siRNAs to identify and destroy homologous mRNAs (7, 8).

In several systems, dsRNA-induced silencing has been harnessed as a powerful tool for the analysis of gene function. Particularly in *Caenorhabditis elegans*, RNAi has emerged as the standard protocol for quickly assessing the consequences of inhibiting gene function. In fact, programs are underway to create RNAi libraries that can be used to suppress, individually, each of the ~19,000 genes in the worm genome (9, 10). In *Drosophila*, the first evidence of dsRNA-induced silencing came from the study of embryos (11), and subsequently, RNAi has proven an effective tool in cultured cells and in adult insects (7, 12, 13).

Despite its utility in diverse systems, harnessing RNA to study gene function in mammals seemed potentially problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs, specifically as an antiviral defense (reviewed in refs. 14 and 15). In somatic cells, dsRNA activates a variety of responses. Predominant among these is PKR, a kinase that is activated by dimerization in the presence of dsRNA (16). PKR, in turn, phosphorylates EIF2α, causing a nonspecific translational shutdown (reviewed in ref. 14). dsRNA also activates 2′-5′ oligoadenylate polymerase, the product of which is an essential cofactor for a nonspecific ribonuclease, RNase L (reviewed in ref. 17).

Recently, Tuschl and colleagues (5) have demonstrated that RNAi can be provoked in numerous mammalian cell lines through the introduction of siRNAs. These siRNAs avoid provoking the PKR response by virtue of their small size and are presumed to be incorporated into the RNAi pathway by mimicking the products of the Dicer enzyme, which catalyzes the initiation step of RNAi (18). The ability to apply RNAi in mammals will undoubtedly spark a firestorm of effort to assess the consequences of suppressing the expression of genes in cultured mammalian cells.

The power of RNAi as a genetic tool would be greatly enhanced by the ability to engineer stable silencing of gene expression. Whereas the production of small RNAs via *in vivo* expression is problematic, stable silencing has been induced in model organisms by directed expression of long dsRNAs (13, 19, 20). We therefore undertook an effort to identify mammalian cells in which long dsRNAs could be used as RNAi triggers in the hope that these same cell lines would provide a platform upon which to develop stable silencing strategies.

Materials and Methods

Cell Culture. P19 mouse embryonic carcinoma cells (American Type Culture Collection, CRL-1825) were cultured in α-MEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL). Mouse embryo stem cells (J1, provided by S. Kim, Cold Spring Harbor Laboratory) were cultured in DMEM containing ESgro (Chemicon) according to the manufacturer's instructions. C2C12 murine myoblast cells (gift of N. Tonks, Cold Spring Harbor

Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein.

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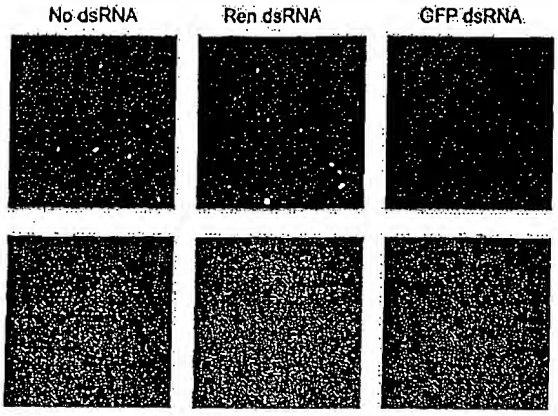

Fig. 1. RNAi in P19 embryonal carcinoma cells. Ten-centimeter plates of P19 cells were transfected by using 5 μg of GFP plasmid and 40 μg of the indicated dsRNA (or no RNA). Cells were photographed by fluorescent and phase-contrast microscopy at 72 h after transfection; silencing was also clearly evident at 48 h posttransfection.

Laboratory) were cultured in DMEM (GIBCO/BRL) supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (GIBCO/BRL).

RNA Preparation. For the production of dsRNA, transcription templates were generated by PCR; they contained T7 promoter sequences on each end of the template (see ref. 7). dsRNAs were prepared by using the RiboMax kit (Ambion, Austin, TX). Firefly and *Renilla* luciferase mRNA transcripts were synthesized by using the Riboprobe kit (Promega) and were gel purified before use.

Transfection and Gene Silencing Assays. Cells were transfected with indicated amounts of dsRNA and plasmid DNA by using FuGENE6 (Roche Biochemicals) according to the manufacturer's instructions. Cells were transfected at 50–70% confluence in 12-well plates containing either 1 or 2 ml of medium per well. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids contain firefly luciferase under the control of SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). These plasmids were cotransfected by using a 1:1 or 10:1 ratio of pGL3-control (250 ng/well) to pRL-SV40. Both ratios yielded similar results. For some experiments, cells were transfected with vectors that direct expression of enhanced green fluorescent protein (EGFP)-US9 fusion protein (21) or red fluorescent protein (pDsRed N1, CLONTECH). RNAi in S2 cells was performed as described (7).

Plasmids expressing hairpin RNAs (RNAs with a self-complimentary stem loop) were constructed by cloning the first 500 bp of the EGFP coding region (CLONTECH) into the FLIP cassette of pRIP-FLIP (E. Bernstein and G.J.H., unpublished data) as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which sports flanking LoxP sites (see Fig. 6*A*). The Zeocin gene (Stratagene), present between the cloning sites, maintains selection and, thus, stability of the FLIP cassette. The FLIP cassette containing EGFP direct repeats was subcloned into pcDNA3 (Invitrogen). To create an inverted repeat for hairpin production, EGFP direct repeat clones were exposed to Cre recombinase (Stratagene) *in vitro* and, afterward, transformed into DL759 *Escherichia coli* (22). These bacteria permit the replication of DNA containing cruciform structures, which tend to form from inverted repeats.

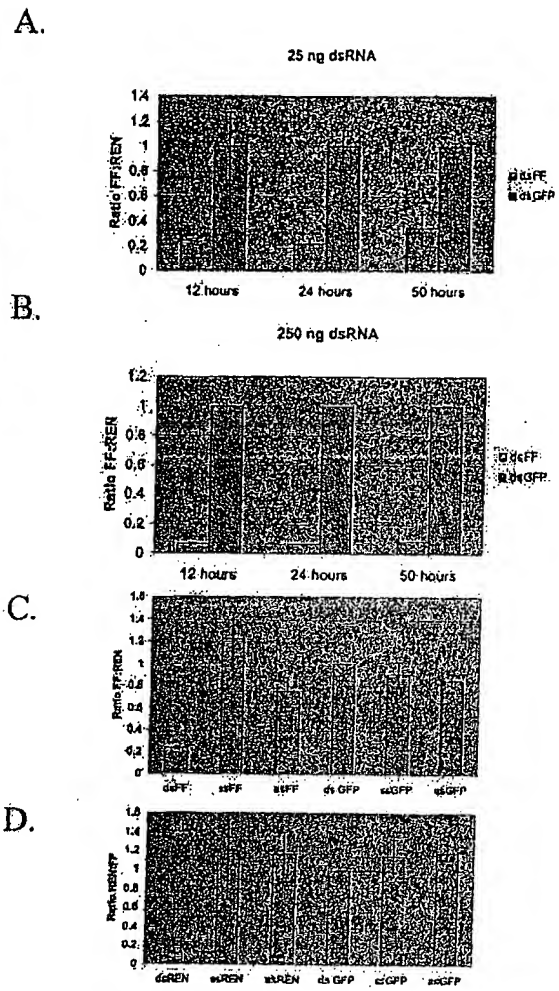

Fig. 2. RNAi of firefly and *Renilla* luciferase in P19 cells. (*A*) P19 cells were transfected with plasmids that direct the expression of firefly and *Renilla* luciferase and dsRNA 500 mers (25 or 250 ng, as indicated), that were either homologous to the firefly luciferase mRNA (dsFF) or nonhomologous (dsGFP). Luciferase activities were assayed at various times after transfection, as indicated. Ratios of firefly to *Renilla* activity are normalized to dsGFP controls. (*B* and *C*) P19 cells in 12-well culture dishes (2 ml of media) were transfected with 0.25 μg of a 9:1 mix of pGL3-Control and pRL-SV40 as well as 2 μg of the indicated RNA. Extracts were prepared 9 h after transfection. (*B*) Ratio of firefly to *Renilla* luciferase is shown. (*C*) Ratio of *Renilla* to firefly luciferase is shown. Values are normalized to dsGFP. The average of three independent experiments is shown; error bars indicate standard deviation.

DL759 transformants were screened for plasmids containing inverted repeats (≈50%).

Silencing of Dicer was accomplished by using a dsRNA comprising exon 25 of the mouse Dicer gene and corresponding to nucleotides 5284–5552 of the human Dicer cDNA.

***In Vitro* Translation and *In Vitro* Dicer Assays.** Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM Hepes, pH 7.3/6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell

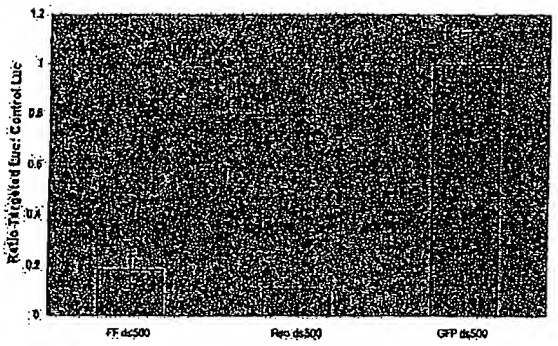

Fig. 3. Specific silencing of luciferase expression by dsRNA in murine embryonic stem cells. Mouse embryonic stem cells in 12-well culture dishes (1 ml of media) were transfected with 1.5 μg of dsRNA along with 0.25 μg of a 10:1 mixture of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 20 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown for FF ds500; the ratio of *Renilla* to firefly is shown for Ren ds500. Both are normalized to ratios from the dsGFP transfection. The average of three independent experiments is shown; error bars indicate standard deviation.

volumes of hypotonic buffer containing *Complete* protease inhibitors (Roche Molecular Biochemicals) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a Dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000 × *g* for 20 min. Supernatants were used in an *in vitro* translation assay containing capped m7G(5′)pppG firefly and *Renilla* luciferase mRNA or in *in vitro* Dicer assays containing ^{32}P-labeled dsRNA. For *in vitro* translation assays, 5 μl of extract were mixed with 100 ng of firefly and *Renilla* mRNA along with 1 μg of dsRNA (or buffer)/10 mM DTT/0.5 mM spermidine/200 mM Hepes, 3.3 mM MgOAc/800 mM KOAc/1 mM ATP/1 mM GTP/4 units of Rnasin/215 μg of creatine phosphate/1 μg of creatine phosphate kinase/1 mM amino acids (Promega). Reactions were carried out for 1 h at 30°C and quenched by adding 1× passive lysis buffer (Promega). Extracts were then assayed for luciferase activity. *In vitro* assays for Dicer activity were performed as described (18).

Construction of Stable Silencing Lines. Ten-centimeter plates of P19 cells were transfected with 5 μg of GFP hairpin expression plasmid and selected for stable integrants by using G-418 (300 ng/ml) for 14 days. Clones were selected and screened for silencing of GFP.

Results

RNAi in Pluripotent Murine P19 Cells. It has long been clear that the nonspecific responses to dsRNA are attenuated during early development. In fact, injection of dsRNA into early-stage mouse embryos can induce sequence-specific silencing of both exogenous and endogenous genes (23, 24). Consistent with the possibility that RNAi might extend to mammals, homologs of the proteins that participate in this response can be easily identified in the mouse and human genomes (reviewed in ref. 4).

We sought to determine whether long dsRNA triggers could induce sequence-specific silencing in cultured murine cells, both to develop this approach as a tool for probing gene function and to allow mechanistic studies of dsRNA-induced silencing to be propagated to mammalian systems. We, therefore, attempted to extend previous studies in mouse embryos (23, 24) by searching for RNAi-like mechanisms in pluripotent, embryonic cell types.

We surveyed a number of cell lines of embryonic origin for the degree to which generalized suppression of gene expression occurred upon introduction of dsRNA. As an assay, we tested the

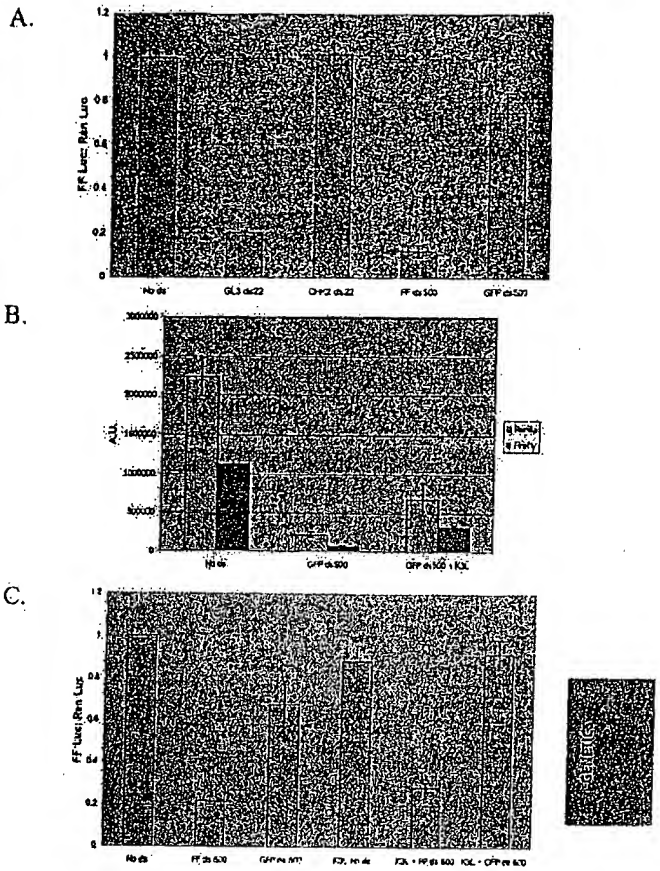

Fig. 4. RNAi in C2C12 murine myoblast cells. (*A*) Mouse C2C12 cells in 12-well culture dishes (1 ml of media) were transfected with 1 μg of the indicated dsRNA along with 0.250 μg of the reporter plasmids pGL3-Control and pRL-SV40. Extracts were prepared and assayed 24 h after transfection. The ratio of firefly to *Renilla* luciferase expression is shown; values are normalized to ratios from the no dsRNA control. The average of three independent experiments is shown; error bars indicate standard deviation. (*B*) C2C12 cells cotransfected with 1 μg of either plasmid alone or a plasmid containing a hyperactive mutant of vaccinia virus K3L (26). The absolute counts of *Renilla* and firefly luciferase activity are shown. (*C*) The ratios of firefly/*Renilla* activity from *B*, normalized to no dsRNA controls.

effects of dsRNA on the expression of GFP as measured *in situ* by counting fluorescent cells. As expected, in both human embryonic kidney cells (293) and mouse embryo fibroblasts, GFP expression was virtually eliminated irrespective of the sequence of the cotransfected dsRNA (not shown). In some pluripotent teratocarcinoma and teratoma cell lines (e.g., NTera1, F9), the PKR response was attenuated but still evident (not shown); however, in contrast, transfection of nonhomologous dsRNAs had no effect on the expression of reporter genes (e.g., GFP, luciferase) either in mouse embryonic stem cells (not shown) or in p19 embryonal carcinoma cells (Fig. 1).

Transfection of P19 embryonal carcinoma cells with GFP in the presence of cognate dsRNA corresponding to the first ≈500 nts of the GFP coding sequence had a strikingly different effect. GFP expression was eliminated in the vast majority of cotransfected cells (Fig. 1), suggesting that these cultured murine cells might respond

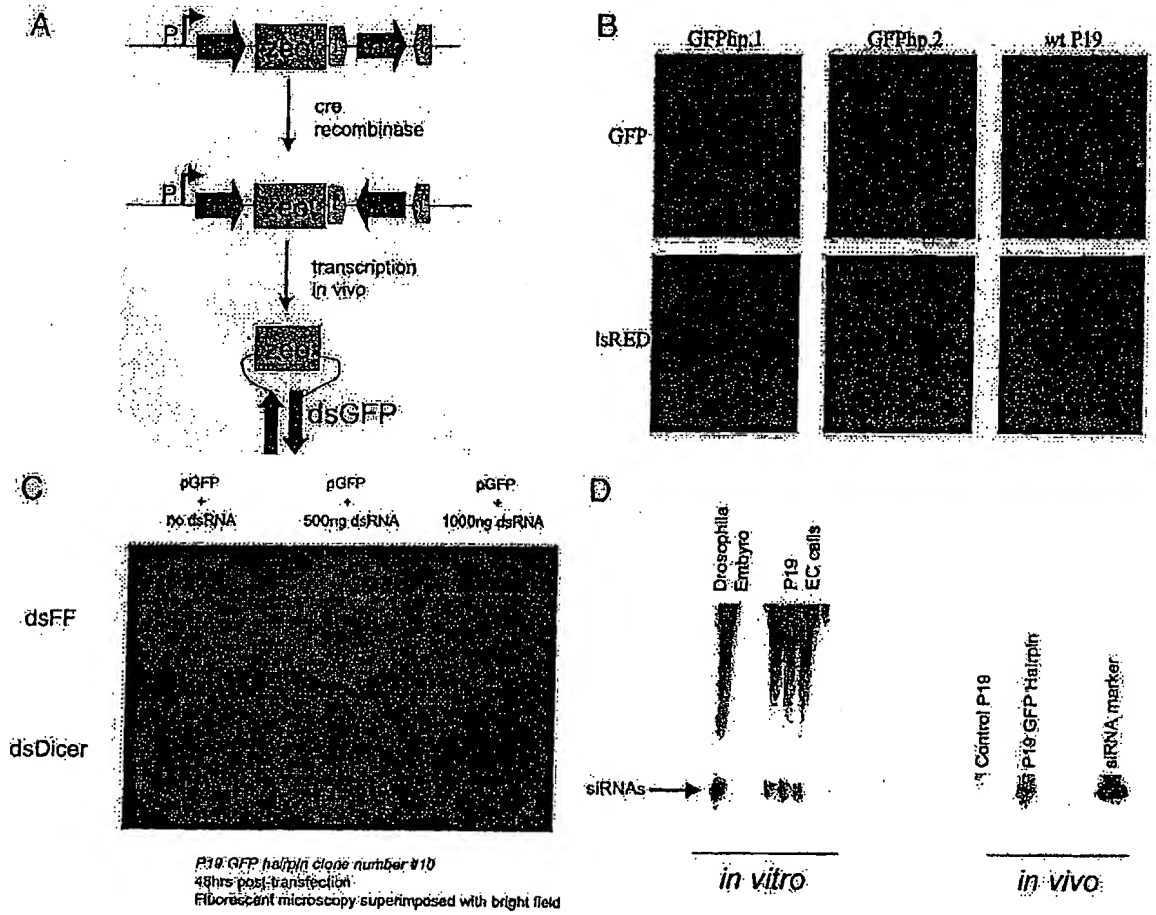

Fig. 5. Expression of a hairpin RNA produces P19 EC cell lines that stably silence GFP. (*A*) A cartoon of the FLIP cassette used to construct the GFP hairpin. GFP represents the first 500 coding base pairs of EGFP. Zeo, zeocin resistance gene; L, Lox; P, the cytomegalovirus promoter in the expression plasmid pcDNA3. Homologous GFP fragments are first cloned as direct repeats into the FLIP cassette. To create inverted repeats for hairpin production, the second repeat is flipped by using Cre recombinase. When transcribed, the inverted repeat forms a GFP dsRNA with a hairpin loop. (*B*) P19 cell lines stably expressing the GFP hairpin plasmid, GFPhp.1 (clone 10) and GFPhp.2 (clone 12), along with wt P19 were transfected with 0.25 μg each of GFP and RFP reporter genes. Fluorescence micrographs were taken by using filters appropriate for GFP and RFP. Magnification is 200×. (*C*) P19 GFPhp.1 cells were transfected with pEGFP and 0, 0.5, or 1 μg of Dicer or firefly dsRNA. Fluorescence micrographs were taken at 48 h posttransfection and are superimposed with bright field images to reveal non-GFP expressing cells. Magnification is 100×. (*D*) *In vitro* and *in vivo* processing of dsRNA in P19 cells. *In vitro* Dicer assays were performed on S2 cells and three independently prepared P19 extracts by using ^{32}P-labeled dsRNA (30°C for 30 min). A Northern blot of RNA extracted from control and GFPhp.1 P19 cells shows the production of ~22mer RNA species in hairpin-expressing cells but not in control cells. Blots were probed with a ^{32}P-labeled "sense" GFP transcript.

to dsRNA in a manner similar to that which we had previously demonstrated in cultured, *Drosophila* S2 cells (7).

To quantify the extent to which dsRNA could induce sequence-specific gene silencing, we used a dual luciferase reporter assay similar to that which had first been used to demonstrate RNAi in *Drosophila* embryo extracts (25). P19 EC cells were transfected with a mixture of two plasmids that individually direct the expression of firefly luciferase and *Renilla* luciferase. These were cotransfected with no dsRNA, with dsRNA that corresponds to the first ~500 nts of the firefly luciferase, or with dsRNA corresponding to the first ~500 nts of GFP as a control. Cotransfection with GFP dsRNA gave luciferase activities that were similar to the no-dsRNA control, both in the firefly/*Renilla* activity ratio and in the absolute values of both activities. In contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to *Renilla* luciferase activity was reduced by up to 30-fold (250 ng, Fig. 2*B*). For comparison, we carried out an identical set of experiments in *Drosophila* S2 cells. Although qualitatively similar results were obtained, the silencing response was more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly luciferase activity to virtually background levels (not shown).

The complementary experiment, in which dsRNA was homologous to *Renilla* luciferase, was also performed. Again, in this case, suppression of the expression of the *Renilla* enzyme was ~10-fold (Fig. 2*D*). Thus, the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pretreatment of the trigger with purified RNase III, a dsRNA-specific ribonuclease, before transfection

greatly reduced its ability to provoke silencing (not shown). Furthermore, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase had little or no effect on expression of the reporters (Fig. 2 *C* and *D*). Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells.

Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml culture media; see Fig. 2*A*). The response was concentration-dependent, with maximal suppression of ≈20-fold being achieved at a dose of 1.5 μg/ml culture media.

Silencing was established rapidly and was evident by 9 h post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 h following a single dose of dsRNA.

RNAi in Embryonic Stem Cells. To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Cotransfection of embryonic stem cells with noncognate dsRNAs (e.g., GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (Fig. 3). However, transfection with either firefly or *Renilla* luciferase dsRNA dramatically and specifically reduced the activity of the targeted enzyme (Fig. 3).

This result suggests that RNAi can operate in multiple murine cell types of embryonic origin, including normal embryonic stem cells. The ability to provoke silencing in a cell type that is normally used for the generation of genetic, mosaic animals suggests the possibility of eventually testing the biological effects of silencing both in culture and in reconstituted animal models.

RNAi in Murine Somatic Cells. RNAi effector pathways are likely to be present in mammalian somatic cells, based on the ability of siRNAs to induce transient silencing (5). Furthermore, we have shown that RNAi initiator and effector pathways clearly exist in embryonic cells that can enforce silencing in response to long dsRNA triggers. We therefore sought to test whether the RNAi machinery might exist intact in some somatic cell lines.

Transfection of HeLa cells with luciferase reporters in combination with long dsRNA triggers caused a nearly complete suppression of activity, irrespective of the RNA sequence. In a murine myoblast cell line, C2C12, we noted a mixture of two responses. dsRNAs homologous to firefly luciferase provoked a sequence-specific effect, producing a degree of suppression that was slightly more potent than was observed upon transfection with cognate ≈21-nt siRNA (ref. 5; Fig. 4*A*). However, with long dsRNA triggers, the specific effect was superimposed upon a generalized suppression of reporter gene expression that was presumably because of PKR activation (Fig. 4*B*).

Numerous mammalian viruses have evolved the ability to block PKR as an aid to efficient infection. For example, adenoviruses express VA RNAs, which mimic dsRNA with respect to binding but not to activation of PKR (16). Vaccinia virus uses two strategies to evade PKR. First is expression of E3L, which binds and masks dsRNAs (26). The second is expression of K3L, which binds and inhibits PKR via its ability to mimic the natural substrate of this enzyme, eIF2α (26).

Transfection of C2C12 cells with a vector that directs K3L expression attenuates the generalized repression of reporter genes in response to dsRNA. However, this protein had no effect on the magnitude of specific inhibition by RNAi (Fig. 4*C*).

These results raise the possibility that, at least in some cell lines and/or cell types, blocking nonspecific responses to dsRNA will enable the use of long dsRNAs for the study of gene function. This might be accomplished through the use of viral inhibitors, as described here, or through the use of cells isolated from animals that are genetically modified to lack undesirable responses.

Stable Suppression of Gene Expression Using RNAi. To date, dsRNAs have been used to induce sequence-specific gene silencing in either cultured mammalian cells or in embryos only in a transient fashion. However, the most powerful applications of genetic manipulation are realized only with the creation of stable mutants. The ability to induce silencing by using long dsRNAs offers the opportunity to translate into mammalian cells work from model systems such as *Drosophila*, plants, and *C. elegans* wherein stable silencing has been achieved by enforced expression of hairpin RNAs (13, 19, 20).

P19 EC cells were transfected with a control vector or with an expression vector that directs expression of a ≈500-nt GFP hairpin RNA from an RNA polymerase II promoter (cytomegalovirus). Colonies arising from cells that had stably integrated either construct were selected and expanded into clonal cell lines. Each cell line was assayed for persistent RNAi by transient cotransfection with a mixture of two reporter genes, dsRED to mark transfected cells and GFP to test for stable silencing.

Transfection of clonal P19 EC cells that had stably integrated the control vector produced equal numbers of red and green cells, as would be expected in the absence of any specific silencing response (Fig. 5*B*), whereas cells that express the GFP hairpin RNA gave a very different result. These cells expressed the dsRED protein with an efficiency comparable to that observed in cells containing the control vector. However, the cells failed to express the cotransfected GFP reporter (Fig. 5*B*). These data provide a strong indication that continuous expression of a hairpin dsRNA can provoke stable, sequence-specific silencing of a target gene.

In *Drosophila* S2 cells and *C. elegans* (18, 27–30), RNAi is initiated by the Dicer enzyme, which processes dsRNA into ≈22-nt siRNAs (18). In both, S2 cells and *C. elegans* experiments by using dsRNA to target Dicer suppress the RNAi response (18, 27, 29). Whether Dicer plays a central role in hairpin-induced gene silencing in P19 cells was tested by transfecting P19 cells stably transfected with GFP hairpin constructs with mouse *Dicer* dsRNA (see *Materials and Methods*). Treatment with *Dicer* dsRNA, but not control dsRNA, resulted in derepression of GFP (Fig. 5*C*).

dsRNA Induces Posttranscriptional Silencing. A key feature of RNAi is that it exerts its effect at the posttranscriptional level by destruction of targeted mRNAs (reviewed in ref. 4). To test

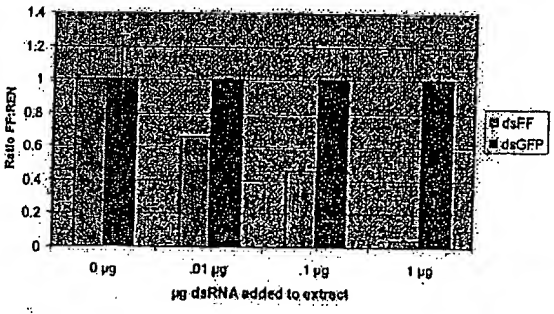

Fig. 6. dsRNA induces silencing at the posttranscriptional level. P19 cell extracts were used for *in vitro* translation of firefly and *Renilla* luciferase mRNA (100 ng each). Translation reactions were programmed with various amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA (dsLUC) or nonhomologous (dsGFP). Luciferase assays were carried out after a 1-h incubation at 30°C. Ratios of firefly to *Renilla* activity are normalized to no dsRNA controls. Standard deviations from the mean are shown.

whether dsRNAs induced silencing in mouse cells via posttranscriptional mechanisms, we used an assay identical to that, used initially to characterize RNAi responses in *Drosophila* embryo extracts (25). We prepared lysates from P19 EC cells that were competent for *in vitro* translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of nonspecific dsRNAs to these extracts had no substantial effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase (Fig. 6). In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the dsRNA had little effect, comparable to a nonspecific dsRNA control, and pretreatment of the dsRNA silencing trigger with RNase III greatly reduced its potential to induce silencing *in vitro*. A second hallmark of RNAi is the production of small, ≈22-nt siRNAs, which determine the specificity of silencing. We found that such RNA species were generated from dsRNA in P19 cell extracts (Fig. 5*D*, *in vitro*), indicative of the presence of a mouse Dicer activity. These species were also produced in cells that stably express GFP hairpin RNAs (Fig. 5*D*, *in vivo*). Considered together, the posttranscriptional nature of dsRNA-induced silencing, the association of silencing with the production of ≈22-nt siRNAs, and the dependence of this response on Dicer, a key player in the RNAi pathway, strongly suggests that dsRNA suppresses gene expression in murine cells via a conventional RNAi mechanism.

Discussion

The discovery that dsRNA could induce gene silencing in organisms as diverse as plants and parasitic protozoans has raised the possibility that RNAi might be a nearly universal mechanism of gene silencing. This notion has been supported by the identification of homologs of proteins that participate in the silencing process in virtually all genomes examined to date, with the exception of *Saccharomyces cerevisiae* (reviewed in ref. 4). The first indications that this response might also extend to mammals came from the observation that injection of dsRNAs into early mouse embryos induced sequence specific silencing (23, 24). Recent work by Tuschl and colleagues (5) had shown that siRNAs can induce silencing in numerous mammalian cell lines, presumably by entering the RNAi pathway. However, both in mouse embryos and previous mammalian cell culture studies, silencing was transient.

As an extension of these pioneering studies, we have demonstrated that dsRNA can induce potent and specific gene silencing in mouse embryonic cell lines. Specifically, we have shown that silencing can be induced by long dsRNAs in mouse embryonal carcinoma cell lines, in normal mouse embryonic stem cells, and in some mouse somatic cells. There are several indications that this phenomenon might be mechanistically related to RNA interference pathways that have been characterized in plants, *C. elegans*, and *Drosophila*. First, induction of silencing requires dsRNA. Second, *in vitro* studies suggest that silencing occurs at the posttranscriptional level. Third, silencing is correlated with the appearance of ≈22-nt siRNAs homologous to the gene that is being suppressed. However, final placement of the phenomenon reported here within the pantheon of dsRNA-induced silencing mechanisms will require a characterization of the protein and/or ribonucleoprotein machinery, which enforces suppression. A significant step toward this goal has been taken by the demonstration that Dicer is required for dsRNA-induced silencing in P19 cells.

We have demonstrated that stable, sequence-specific silencing can be induced by enforcing endogenous expression of RNA hairpins. The ability to create permanent cell lines with a desired loss-of-function phenotype extends the utility of RNAi as method for probing gene function in mammalian cells. This capability enables the production of large numbers of silenced cells for biochemical analysis and permits the evaluation of phenotypes over long time spans. However, perhaps the two most important ramifications of stable RNAi are the ability to harness this technology for unbiased, phenotype-based genetic selections and the possibility that stably silenced, embryonic cell lines might ultimately be used to reconstitute animals containing a specifically silenced locus.

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articles

Specific interference with gene function by double-stranded RNA in early mouse development

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The use of double-stranded (ds) RNA is a powerful way of interfering with gene expression in a range of organisms, but doubts have been raised about whether it could be successful in mammals. Here, we show that dsRNA is effective as a specific inhibitor of the function of three genes in the mouse, namely maternally expressed c-*mos* in the oocyte and zygotically expressed *E-cadherin* or a *GFP* transgene in the preimplantation embryo. The phenotypes observed are the same as those reported for null mutants of the endogenous genes. These findings offer the opportunity to study development and gene regulation in normal and diseased cells.

To study early developmental events in the embryo, it is often desirable to be able to eliminate expression of a specific gene. The most valuable information would be obtained if the function of the gene of interest could be disturbed in specific cells of the embryo and at defined times. In such a situation, in the mouse, the classical techniques of gene 'knockout' cannot be used, because they eliminate gene function universally throughout the embryo. Furthermore, if a gene is repeatedly used in space and time to direct developmental processes, elimination of its role by gene 'knockout' may deny an understanding of everything but the first event. Even when the aim is to study the very first time in development at which a gene functions, the contribution of maternal transcripts and their translation products can mask the effects of the gene knockout.

There are, nevertheless, many instances in which the existing 'knockout' technology is extremely powerful. It is, however, extremely laborious. It necessitates, first, making a disrupted gene segment that is suitably marked to enable the selection of homologous recombination events in cultured embryonic stem cells. Such cells must then be incorporated into blastocysts and the resulting chimaeric animals used to establish pure breeding lines before homozygous mutants can be obtained.

Some of these difficulties could be overcome if a method for double-stranded-RNA interference (RNAi) of gene expression could be developed for mammalian cells. Such an approach, first developed in *Caenorhabditis elegans*[1], has also been shown to be

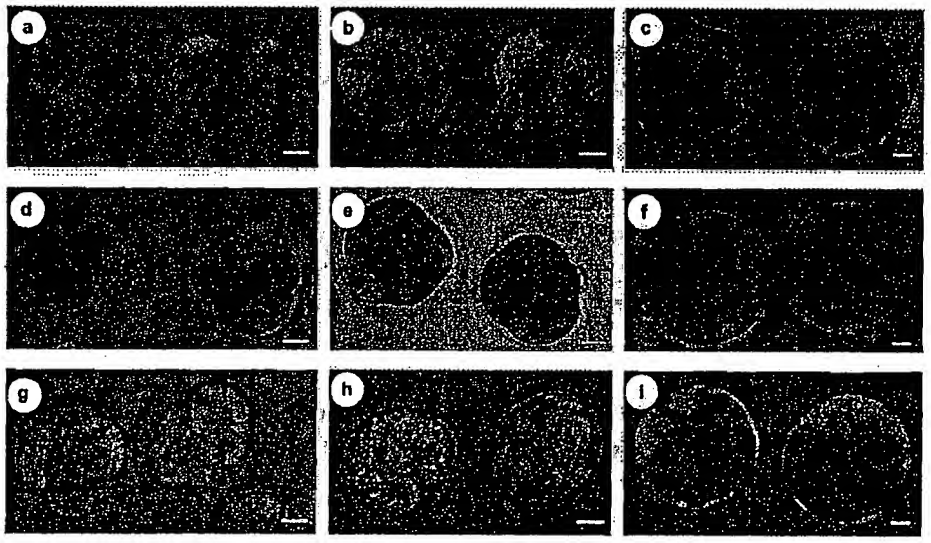

Figure 1 *MmGFP* dsRNA specifically abrogates MmGFP expression in *MmGFP* transgenic embryos. a–c, Representative embryos out of 131 *MmGFP* transgenic embryos obtained from 11 different matings between F_1 females and *MmGFP* transgenic males. a, Four- to six-cell embryos; b, morulae; c, blastocysts. A similar pattern of GFP expression was obtained after injection of antisense *MmGFP* RNA. d–f, Representative embryos out of 147 *MmGFP* transgenic embryos that had been injected with *MmGFP* dsRNA at the one-cell stage. d, Four- to six-cell embryos; e, morulae; f, blastocysts. g–i, Representative embryos out of 18 *MmGFP* transgenic embryos that had been injected with c-*mos* dsRNA at the one-cell stage. g, Six-cell-stage embryos; h, morulae; I, blastocysts. Scale bars represent 20μm.

effective in other eukaryotes, including *Drosophila melanogaster*[2], *Trypanosoma brucei*[3], planarians[4] and plants[5]. The application of this approach has also been demonstrated in zebrafish embryos, but with limited success[6]. So far there has been no report that RNAi can be used in mammals. Moreover, there are several indications of potential limitations to its function in this group of animals. Principal among these is that the accumulation of very small amounts of dsRNA in mammalian cells following viral infection results in the interferon response, which leads to an overall block to translation and the onset of apoptosis[7]. Such considerations have discouraged investigators from using RNAi in mammals.

Two factors motivated us to attempt the use of RNAi as a means of eliminating specific gene expression in the mouse embryo. First, we have developed approaches for microinjection of synthetic messenger RNAs into both mouse oocytes and preimplantation embryos as a means of successfully directing gene expression[8,9]. Second, we have established a transgenic line of mice expressing a modified form of the green fluorescent protein (MmGFP) from the ubiquitous elongation factor-1α (EF1α) promoter[10] that could provide a rapid visual assay for the effective elimination of expression of this marker gene. This facilitated assessment of whether RNAi could be effective in the mouse. Here we show that it is possible to interfere with specific gene expression in the mouse oocyte and zygote following microinjection of the appropriate dsRNA. We show that RNAi can phenocopy the effects of disrupting the maternal expression of the *c-mos* gene in the oocyte, preventing the arrest of meiosis at metaphase II. It also interferes with the zygotic expression of E-cadherin, disrupting development of the blastocyst, as also observed in the corresponding knockout mice. These studies show that RNAi can be effective in mammalian cells and this fact should have substantial implications for the analysis of gene function.

Results

dsRNA prevents expression of a *GFP* transgene. To determine whether dsRNA might be used to prevent gene expression in the mouse embryo, we developed an experimental test system using a transgenic strain of mice that expresses MmGFP under the control of the EF1α promoter[10]. This line offered the advantage that GFP expression can be easily visualized in living embryos, and, because its function is non-essential, we could monitor any non-specific deleterious effects of dsRNA on embryonic development. To avoid the complication of endurance of maternal gene products, we used heterozygous embryos in which the transgene was paternally derived. The onset of GFP expression in these embryos is seen by the appearance of green cells following the initiation of zygotic transcription at the two-cell stage.

The injection of *MmGFP* dsRNA into the single-cell zygote prevented the onset of the appearance of green fluorescence at the two- to four-cell stages. After injection, embryos were cultured *in vitro* for 3–4 days to the blastocyst stage. While uninjected embryos expressed MmGFP in the expected manner (Fig. 1a–c), all embryos injected with *MmGFP* dsRNA showed a markedly reduced green fluorescence throughout this period (Fig. 1d–f), with a minor proportion (6.8%) showing residual weak fluorescence. The embryos injected with *MmGFP* dsRNA showed normal preimplantation

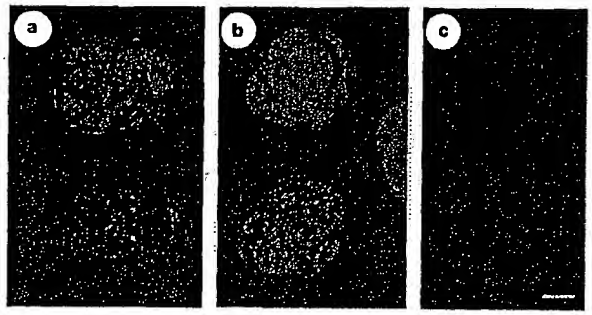

Figure 2 **Interference with expression of injected synthetic *MmGFP* mRNA.** **a–c**, Wild-type morulae injected at the one-cell stage with **a**, *MmGFP* mRNA alone; **b**, *MmGFP* mRNA together with *E-cadherin* dsRNA; **c**, *MmGFP* mRNA together with *MmGFP* dsRNA. Scale bar represents 20 μm.

Table 1 Phenotypes obtained by injection of *E-cadherin* dsRNA into zygotes

Experiment	No. of embryos developing into blastocysts after injection of *MmGFP* dsRNA*	No. of embryos forming a cavity after injection of *E-cadherin* dsRNA†
1	16/21	5/21
2	7/8	9/20
3	12/24	6/60
4	10/14	5/10
5	21/22	10/19
Total	66/89 (74±5%) formed expanded blastocysts	35/130 (27±4%) formed a cavity (but did not form expanded blastocysts). The remainder failed to develop to this stage

240 uninjected zygotes were also studied. Of these, 91.6±1.1% (mean±s.e.m.) formed expanded blastocysts.

* *MmGFP* dsRNA (2 mg ml^{-1}) was injected as a control.

† 2mg ml^{-1} *E-cadherin* dsRNA were injected.

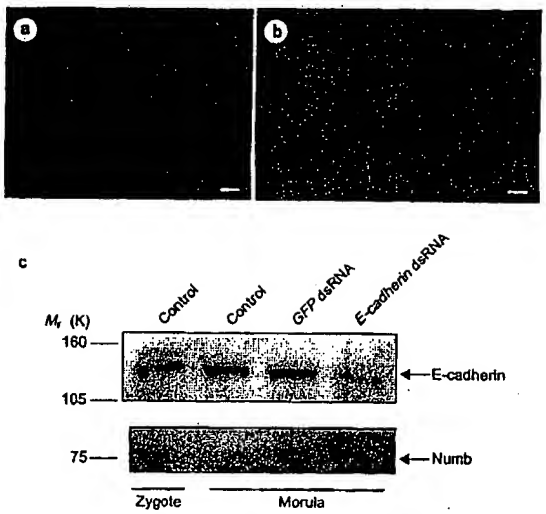

Figure 3 **Injection of *E-cadherin* dsRNA to the zygote reduces E-cadherin expression and perturbs the development of the injected embryos.** **a**, Immunofluorescent staining of E-cadherin in embryos injected at the one-cell stage with *MmGFP* dsRNA, and cultured for 4 days *in vitro* until the blastocyst stage. **b**, Immunofluorescent staining of E-cadherin in embryos injected at the one-cell stage with *E-cadherin* dsRNA, and cultured for 4 days *in vitro*. Note the altered development of these embryos. Scale bars represent 20μm. **c**, Western blot analysis of E-cadherin expression in zygotes, uninjected (control) morulae (collected at the one-cell stage and cultured *in vitro* for 3 days), morulae injected at the one-cell stage with 2mg ml^{-1} *GFP* dsRNA and cultured *in vitro* for 3 days, and morulae injected at the one-cell stage with 2mg ml^{-1} *E-cadherin* dsRNA and cultured *in vitro* for 3 days. The expression of Numb protein is shown as a loading control. In each case, proteins were extracted from 15 embryos. This experiment was repeated three times with the same result. The reduction of signal following injection of *E-cadherin* dsRNA was 6.5±1-fold (mean ±s.d.).

Table 2 Phenotypes observed after injection of c-mos dsRNA into germinal-vesicle-stage oocytes

dsRNA injected	Experiment	Number of oocytes undergoing Spontaneous activation	Fragmentation	Known null mutant phenotype
MmGFP (2 mg ml^{-1})	1	0/21	0/21	NA*
	2	0/22	2/22	
	3	1/17	0/17	
	4	0/13	0/13	
	Total	1/73 (1.4±1.4%)	2/73 (2.7±1.9%)	
c-*mos* (2 mg ml^{-1})	1	15/32	11/32	60–75% released from the metaphase-II arrest. High degree of cytoplasmic fragmentation[11,15]
	2	12/22	0/22	
	3	20/40	2/40	
	4	6/14	2/14	
	Total	53/108 (49.1±5%)	15/108 (13.9±3.3%)	
c-*mos* (0.1 mg ml^{-1})	1	4/17	0/17	As above
	2	8/16	2/16	
	Total	12/33 (36 ± 8%)	2/33 (6.1 ± 4.2%)	

* NA, not applicable. Uninjected oocytes underwent spontaneous activation rarely and at a similar frequency to those injected with *MmGFP* dsRNA. Of 158 uninjected oocytes, 1.3% showed spontaneous activation and 3.8% underwent fragmentation. Totals are shown as mean percentages ± s.e.m.

development *in vitro* (Fig. 1d–f). When transferred into pseudopregnant females, they were also able to implant at the same frequency as embryos derived from uninjected zygotes (40.9% and 36.1%, respectively). We compared 18 injected embryos with 22 uninjected controls at two different postimplantation stages to determine whether they underwent normal development. Injected embryos developed into normal gastrulating embryos at 7.0 days post-coitum (d.p.c.), and were indistinguishable from control uninjected embryos (data not shown). At 8.5 d.p.c. (three- to four-somite stage) the injected embryos were also morphologically normal, showing that the injection of dsRNA is not toxic.

The interference with gene expression was specific, as shown by the fact that injection of an unrelated dsRNA, corresponding to a segment of the c-*mos* transcript, into MmGFP transgenic embryos did not result in a decrease in green fluorescence (Fig. 1g–i). The injection of c-*mos* dsRNA did not perturb the development of the embryos, consistent with the previous finding that the c-*mos* gene is not required for normal embryonic development[11]. Similarly, injection of dsRNA corresponding to a segment of the *E-cadherin* transcript into transgenic zygotes (59 embryos observed) did not result in a decrease in green fluorescence and did not shut down protein synthesis, although the phenotype of such embryos was abnormal (data not shown; see also below). Transgenic zygotes injected with antisense *MmGFP* RNA retained the green fluorescence at all preimplantation stages (37 embryos observed) (data not shown).

We attempted to determine whether expression of MmGFP from injected capped full-length *MmGFP* mRNA could be eliminated by the co-injection of *MmGFP* dsRNA. We found that green fluorescence was greatly diminished or abolished in such injected embryos (Fig. 2c). This was in contrast to embryos injected with sense *MmGFP* mRNA, or co-injected with both sense *MmGFP* mRNA and the 'irrelevant' dsRNA for *E-cadherin* (Fig. 2a, b). Thus dsRNA can interfere with the expression of both a chromosomally located gene, and of synthetic mRNA introduced by microinjection.

Phenocopying an *E-cadherin* knockout. We then assessed the specific developmental consequences of injecting *E-cadherin* dsRNA. E-cadherin is both maternally and zygotically expressed during preimplantation development. Disruption of the *E-cadherin* gene, using homologous recombination to remove regions of the molecule esssential for adhesive function, leads to a severe preimplantation defect. These embryos can initially undergo compaction, as a result of the presence of maternally expressed E-cadherin. However, they show a defect in cavitation and never form normal blastocysts[12,13].

Following injection of *E-cadherin* dsRNA, the phenotype was identical to that of the null mutant embryos[12]. Thus, the embryos initially developed normally to the compaction stage of the morula (data not shown). However, 70% of them never formed a cavity. The remaining 30% formed a cavity, but never developed into normal blastocysts (Table 1, Figs 3b, 4). In contrast, the majority of uninjected embryos or control embryos injected with *MmGFP* dsRNA cavitated and formed normal blastocysts (Table 1, Fig. 3a).

Analysis of E-cadherin expression by immunostaining and immunoblotting showed that the expression of E-cadherin was dramatically decreased after injection of *E-cadherin* dsRNA (Fig. 3b, c). In contrast, no decrease in E-cadherin expression was observed in the embryos injected with *MmGFP* dsRNA, in which the level of E-cadherin expression was similar to that of the control uninjected embryos (Fig. 3c). The level of E-cadherin at the morula stage in embryos injected with *E-cadherin* dsRNA was lower than in newly fertilized embryos before injection (Fig. 3c). This residual E-cadherin protein may largely reflect persistence of maternally expressed protein whose synthesis ceases during the two-cell stage[14]. This residual maternal protein is present until the late blastocyst stage in

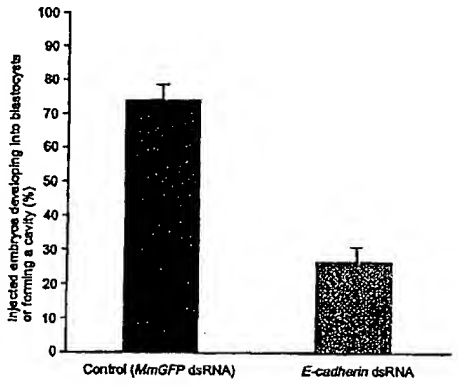

Figure 4 **Incidence of cavity formation after injection of *E-cadherin* dsRNA into the zygote.** Graphical representation of the results shown in Table 1. Dark grey, the percentage of embryos developing into blastocysts following injection of control *MmGFP* dsRNA. Light grey, the percentage of embryos forming a cavity after injection of *E-cadherin* dsRNA. Standard error bars are indicated.

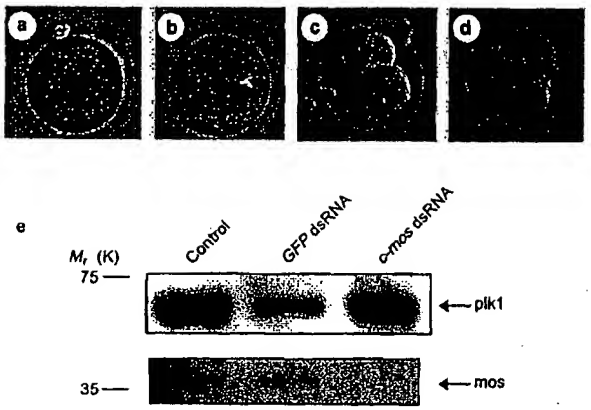

Figure 5 **Injection of c-*mos* dsRNA into immature oocytes inhibits c-mos expression and causes parthenogenetic activation. a–d**, Examples of parthenogenetically activated eggs obtained after injection of c-*mos* dsRNA in germinal-vesicle-stage oocytes. **a**, Control oocyte arrested in metaphase II; **b**, one-cell embryo (white arrow indicates the pronucleus); **c**, two-cell embryo; **d**, four-cell embryo. Scale bar represents 20 μm. **e**, Western blot analysis of c-mos expression in (left to right): oocytes arrested in metaphase II; oocytes injected at the germinal-vesicle stage with 2 mg ml^{-1} *MmGFP* dsRNA and cultured *in vitro* for 12 h; and oocytes injected at the germinal-vesicle stage with 2 mg ml^{-1} c-*mos* dsRNA and cultured *in vitro* for 12 h. In each case, proteins were extracted from 35 oocytes. The expression of polo-like kinase 1 (plk1) is shown as a loading control. This experiment was repeated three times with the same result.

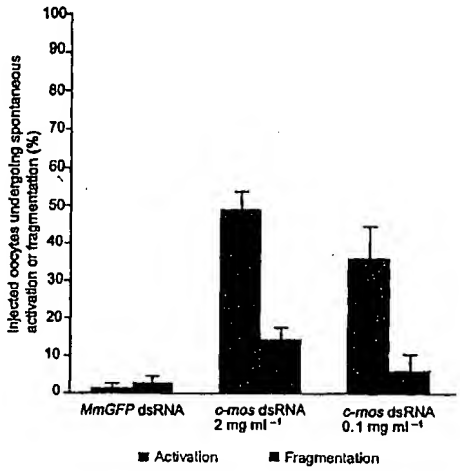

Figure 6 **Incidence of spontaneous activation and fragmentation after injection of c-*mos* dsRNA into the germinal-vesicle-stage oocyte.** Graphical representation of the results shown in Table 2. Oocytes were injected with the indicated dsRNAs. The percentage of injected oocytes undergoing spontaneous activation is shown in light grey, and the percentage undergoing fragmentation in dark grey. Standard error bars are indicated.

homozygous null embryos[12].

We conclude that injection of *E-cadherin* dsRNA leads to a striking reduction in amounts of E-cadherin protein and, consequently, a similar phenotype to that of the null mutant embryos.

dsRNA interference in the oocyte. To determine whether dsRNA might be used to interfere with maternally expressed genes, we sought a model gene producing a characteristic knockout phenotype. C-mos is an essential component of cytostatic factor, which is responsible for arresting the maturing oocyte at metaphase in the second meiotic division. In c-*mos*$^{-/-}$ mice, between 60% and 75% of oocytes do not maintain this metaphase-II arrest and instead initiate parthenogenetic development[11,15]. C-*mos* mRNA is present in fully grown immature oocytes, and its translation is initiated from maternal templates when meiosis resumes following germinal-vesicle breakdown[16]. Thus, injection of c-*mos* dsRNA would allow us to test whether dsRNA could interfere with maternal mRNA expression.

When we injected c-*mos* dsRNA into oocytes, about 63% did not maintain arrest in metaphase II (Table 2, Figs 5, 6). Of these, 78% initiated parthenogenetic development and progressed to two- to four-cell stage embryos (Fig. 5b–d). The remainder underwent fragmentation. Both of these events occur at similar frequencies in null mutant oocytes[11]. In contrast, only 1–2% of control oocytes, either uninjected or injected with *MmGFP* dsRNA, underwent spontaneous activation (Table 2, Fig. 6). 42% of injected oocytes failed to undergo metaphase-II arrest when we reduced the concentration of injected c-*mos* dsRNA by 20-fold (Table 2). We confirmed that c-*mos* dsRNA interferes with c-*mos* expression by immunoblot analysis carried out 12 h after the injection of germinal-vesicle-stage oocytes, before the phenotypic consequences of its loss of expression become apparent (Fig. 5e). Thus, injection of c-*mos* dsRNA into the oocyte specifically interferes with c-*mos* activity, mimicking the targeted deletion of c-*mos* by homologous recombination. These experiments show that dsRNA is able to block the expression of maternally provided gene products.

Discussion

We have shown that dsRNA can be used as a specific inhibitor of gene activity in the mouse oocyte and preimplantation embryo. We showed the specificity of the procedure by individually inhibiting the expression of three different genes: c-*mos* in the oocyte, and *E-cadherin* or a *GFP* transgene in the early embryo. In the cases of the two endogenous mouse genes, this results in phenotypes comparable to those of null mutants. Our experiments aimed at preventing expression of the *GFP* transgene indicate that RNAi *per se* does not affect the normal course of development.

Thus it appears that the concerns that RNAi might not work in the mouse may have been raised prematurely (reviewed in ref. 17). Concern has been expressed that the protocols used for invertebrate and plant systems are unlikely to be effective in mammals, because accumulation of dsRNA in mammalian cells can result in a general blockage of protein synthesis. The presence of extremely low concentrations of dsRNA in viral infections triggers the interferon response[18], part of which is the activation of a dsRNA-responsive protein kinase (PKR)[19]. This enzyme phosphorylates and inactivates translation factor EIF2α in response to dsRNA. The consequence is a global suppression of translation, which in turn triggers apoptosis. However, we have shown here that the injection of a dsRNA is specific to the corresponding gene; it does not cause a general translational arrest, because embryos continue to develop and we see no signs of cell death. It is possible that the early mouse embryo is incapable of an interferon response, and that there may still be difficulties in using RNAi at later stages. However, the interferon response normally occurs in response to viral infection and is usually induced experimentally using synthetic double-stranded ribonucleotide homopolymers. 'Natural' dsRNA may be less effective at inducing PKR, and the degree of induction could vary between cell types, in which case RNAi would be effective.

It has been suggested in other systems that genetic interference from injected sense or antisense RNA is actually mediated by dsRNA present at a low level in all *in vitro* RNA syntheses because of the nonspecific activity of RNA polymerases[1]. Antisense RNA has been used as a means of reducing gene expression in the embryos of a number of species. Although it has had considerable success in *Drosophila*, it has been disappointing in *Xenopus*, zebrafish and

mouse embryos. In *Xenopus*, the limitations in using the antisense approach were thought to be due to a prominent RNA-melting activity[20,21], exerted by the dsRNA-specific adenosine deaminase (dsRAD), and which itself argues against the likelihood of success for RNAi. However, although dsRAD has the potential to lead to the instability of injected dsRNA and thereby might be expected to reduce the efficacy of the approach, others have postulated that dsRNA modifed by this enzyme might actually mediate RNAi through the targeted degradation of endogenous RNA[17,22]. In the mouse embryo, the use of antisense RNA has had inconsistent and limited success in reducing gene expression, possibly because of the instability of RNA, particularly between the two–four-cell stages[23]. It has been recently reported that dsRNA might be more effective than antisense RNA in inhibiting gene expression in zebrafish embryos[6]. However, in contrast to our experiments in the mouse, these initial experiments with zebrafish embryos indicated only partial success of dsRNA interference.

Two of our experiments support the hypothesis that RNAi acts in the mouse by either inducing degradation of the targeted RNA or inhibiting its translation. First, we showed that injection of *MmGFP* dsRNA inhibits the expression of co-injected sense *MmGFP* mRNA. Second, we injected dsRNA against c-*mos* into oocytes before the germinal vesicle breaks down, the stage when c-*mos* mRNA has accumulated but has not yet been translated. C-*mos* is translated when the germinal vesicle breaks down, to arrest oocytes in metaphase of the second meiotic division[16,24]. We found that c-mos dsRNA prevents c-mos function: oocytes proceed through metaphase II and undergo parthenogenetic activation. In each case, the effects of RNAi persist for sufficient time to phenocopy the loss of gene function. As interfering with the expression of MmGFP is of no consequence to the embryo, this allows us to determine how long the RNAi effect persists. We found that although green fluorescence was absent or greatly reduced by *MmGFP* dsRNA in transgenic blastocysts injected as zygotes, fluorescence did return in embryos at 6.5 days postimplantation. During this time period, some 10–20 cells of the inner cell mass undergo a 100-fold increase in cell number, corresponding to a 40–50-fold increase in cell mass. This is coincident with a parallel increase in the expression of the transgene in uninjected embryos.

As the effects of dsRNA-mediated inhibition of gene expression persist for more than six rounds of cell division, RNAi offers new opportunities to study loss-of-function phenotypes in specific cells and at specific stages of development of the early mouse embryo. It should be possible to study the loss of function of not only any single gene, but also combinations of genes, either family members that may have redundant functions, or several members of a regulatory pathway. Moreover, the use of RNAi can be extended to evaluate the loss of function of particular genes during oocyte maturation. This also provides a means of eliminating the expression of maternally provided RNA to study maternal effects of genes that show lethality in homozygous mutants. We anticipate that it should be equally effective in other mammals, including both domestic animals and humans, in which it is difficult or impossible (because unethical) to create loss-of-function mutants and perform standard *in vivo* mutational analysis. If the approaches that we describe can be extended to the adult organism, they will have considerable therapeutic power in inhibiting gene activity in several types of disease. At the moment, in addition to allowing the analysis of genes that regulate development, elimination of gene function by RNAi in the mouse oocyte and preimplantation embryo should find widespread application in the study of genes that regulate all basic cellular processes, such as cell–cell interactions, intracellular trafficking and the cell-division cycle. □

Methods

Collection and culture of oocytes and embryos.

Immature oocytes arrested at prophase I of meiosis were collected from ovaries of 4–6-week-old F_1 (CBA × C57/Bl) mice in FHM medium (Speciality Media Inc., Lavalette, NJ) supplemented with bovine serum albumin (BSA) (4 mg ml^{-1}).

F_1 female mice were superovulated by intraperitoneal injections of pregnant mare's serum gonadotrophin (PMSG, 5 international units (I.u.)) and human chorionic gonadotrophin (hCG, 5 i. u.) 48–52 h apart. Fertilized one-cell embryos were obtained from mated females 20–24h after hCG injection.

RNA synthesis and microinjections.

The templates used for RNA synthesis were linearized plasmids. Full-length *MmGFP* complementary DNA (714 base pairs (bp)) was cloned into the T7TS plasmid. A *KpnI/HindIII* fragment of c-*mos* cDNA (550 bp) was cloned into Bluescript pSK. A cDNA fragment corresponding to exons 4–8 of *E-cadherin* (580 bp) was cloned into Bluescript pKS. RNAs were synthesized using the T3 or T7 polymerase, using the Megascripts kit (Ambion). DNA templates were removed with DNase treatment. The RNA products were extracted with phenol/chloroform, and ethanol-precipitated.

To anneal sense and antisense RNAs, equimolar quantities of sense and antisense RNAs were mixed in the annealing buffer (10mM Tris, pH7.4, 0.1mM EDTA) to a final concentration of 2 μM each, heated for 10min at 68 °C, and incubated at 37 °C for 3–4h. To avoid the presence of contaminating single-stranded RNA in the dsRNA samples, the preparations were treated with 2 μg ml^{-1} RNase T1 (Calbiochem) and 1 μg ml^{-1} RNase A (Sigma) for 30min at 37 °C. The dsRNAs were then treated with 140 μg ml^{-1} proteinase K (Sigma), phenol/chloroform-extracted and ethanol-precipitated. Formation of dsRNA was confirmed by migration on an agarose gel: for each dsRNA, the mobility on the gel was shifted compared to the single-stranded RNAs. For comparison of antisense and double-stranded RNAs, equal masses of RNA were injected.

RNAs were diluted in water, to a final concentration of 2–4 mg ml^{-1}. The range of effective concentrations is best illustrated by the c-*mos* RNAi experiment (Table 2) because of the sensitivity of this biological phenotype. The mRNAs were microinjected into the cytoplasm of the oocytes or embryos, using a constant flow system (Transjector, Eppendorf) as described[16]. Each oocyte or embryo was injected with ~10 pl dsRNA. Improved penetrance was achieved by using negative capacitance. After microinjection, oocytes and embryos were cultured in KSOM (Speciality Media Inc.) medium supplemented with 4 mg ml^{-1} BSA, at 37 °C in a 5% CO_2 atmosphere. *MmGFP* transgenic embryos were observed by confocal microscopy (Biorad 1024 scanning head on a Nikon Eclipse 800 microscope). Some blastocysts derived from uninjected zygotes or zygotes injected with *MmGFP* dsRNA were transferred into the uteri of pseudopregnant mothers that had been mated 2.5 days earlier with vasectomized males. Embryos were recovered either at embryonic day (E) 7.0 or E8.5, counting noon of the plug day of the pseudopregnant recipient as E0.5. They were observed by confocal microscopy as described[19].

Immunoblot and immunostaining analysis.

For immunoblot analysis, samples were subjected to SDS–PAGE and proteins were transferred to a hybond nitrocellulose membrane (Amersham). Membranes were preincubated in TBST buffer (20 mM Tris-HCl, pH8.2, 150 mM NaCl, 0.1% Tween-20) containing 5% (*w/v*) non-fat dried milk overnight, to block nonspecific binding of antibodies. They were then incubated with the anti-E-cadherin antibody (DECMA-1), the anti-mos antibody (SantaCruz Biotechnology), the anti-Numb antibody (provided by R. Pedersen), or the anti-plk1 antibody[9] for 1 h, washed in TBST, incubated with the peroxidase-conjugated secondary antibody (SantaCruz Biotechnology) for 1 h, and washed again in TBST. The antibodies were diluted in TBST containing 5% (*w/v*) non-fat dried milk. The secondary antibody was detected by enhanced chemiluminescence (Amersham). The decrease in E-cadherin expression was quantified by comparing the optical density of the bands obtained in each western blot analysis, on a Macintosh computer using the public-domain NIH Image program.

For whole-mount immunofluorescence with anti-E-cadherin antibody, embryos were fixed in 2% paraformaldehyde for 20min at room temperature, followed by permeabilization with 0.1% Triton X-100 for 10min. After preincubation in 2% BSA in PBS for 30 min, embryos were incubated with the anti-E-cadherin antibody for 1 h at 37 °C, and with a Texas-red-conjugated goat anti-rat antibody (Jackson ImmunoResearch) for 1 h at 37 °C. Embryos were observed using the Biorad 1024 laser scanning confocal microscope.

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EXHIBIT 7

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Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference

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SUMMARY

Specific mRNA degradation mediated by double-stranded RNA (dsRNA), which is termed RNA interference (RNAi), is a useful tool with which to study gene function in several systems. We report here that in mouse oocytes, RNAi provides a suitable and robust approach to study the function of dormant maternal mRNAs. *Mos* (originally known as c-*mos*) and tissue plasminogen activator (tPA, *Plat*) mRNAs are dormant maternal mRNAs that are recruited during oocyte maturation; translation of *Mos* mRNA results in the activation of MAP kinase. dsRNA directed towards *Mos* or *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNAs. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity and can result in parthenogenetic activation. *Mos* dsRNA, therefore, faithfully phenocopies the *Mos* null mutant. Targeting the *Plat* mRNA with *Plat* dsRNA results in inhibiting production of tPA activity. Finally, effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively.

Key words: Maternal mRNA, RNA interference, mouse oocyte, *Mos*, *Plat*

INTRODUCTION

'Omne vivum ex ovo' (All living things come from eggs), which is attributed to William Harvey, is probably the first articulation of the current view that the program for early development is established during oogenesis. During oogenesis in the mouse, oocytes grow and acquire the ability to resume and complete meiosis (acquisition of meiotic competence) (Sorensen and Wassarman, 1976; Wickramasinghe et al., 1991), as well as the ability to be fertilized and develop to term (acquisition of developmental competence) (Eppig and O'Brien, 1996). Meiotic maturation and egg activation are accompanied by the recruitment of many maternal mRNAs (Schultz et al., 1979; Schultz and Wassarman, 1977; Van Blerkom, 1981), and presumably some of these direct the synthesis of proteins that are required for the formation of a fertilizable egg that is capable of developing to term. One such mRNA is the *Mos* mRNA. The mobilization of the *Mos* mRNA results in the ultimate activation of mitogen-activated protein (MAP) kinase, whose activity is required to maintain arrest at metaphase II (Gebauer and Richter, 1997; Sagata, 1997); oocytes lacking the *Mos* gene mature to metaphase II but then undergo spontaneous activation, i.e., they emit the second polar body and form a pronucleus (Colledge et al., 1994; Hasimoto et al., 1994). The tissue plasminogen activator (tPA, *Plat*) mRNA is another maternal mRNA that is recruited during oocyte maturation (Huarte et al., 1987; Vassalli et al., 1989). Although tPA is synthesized during maturation and secreted following fertilization, and then becomes associated with a cell-surface receptor on the embryo (Carroll et al., 1993), *Plat* knockout mice are viable and fertile, but do display mild perturbations in phenotype, e.g., retardation in neuronal migration (Seeds et al., 1999).

To date, an antisense RNA approach has been the most widely used method to assess the function of maternal mRNAs that are recruited during oocyte maturation. Nevertheless, this approach has problems. For example, an antisense RNA approach has been used to assess the role of *Mos* mRNA recruitment during maturation. The phenotypes observed range from permitting germinal vesicle breakdown but inhibiting emission of the first polar body (Paules et al., 1989; Zhao et al., 1991), to emission of the first polar body but entering interphase instead of proceeding to and arresting at metaphase II (O'Keefe et al., 1989). In contrast, the phenotype of a *Mos* null mutant generated by homologous recombination is that the oocytes proceed to metaphase II, but meiotic arrest is not maintained and the eggs spontaneously undergo parthenogenetic activation (Colledge et al., 1994; Hasimoto et al., 1994). This discrepancy between the phenotypes observed by the antisense approach with that of a 'true' knockout potentially confounds the use of antisense RNA to study the function of a dormant maternal mRNA. Antisense RNA can

also target and destroy the *Plat* mRNA. The efficacy of destruction of the untranslated *Plat* mRNA, however, appears restricted to antisense RNA directed towards the 3′ UTR (Strickland et al., 1988). Antisense RNAs directed at other portions of the *Plat* mRNA are far less effective and can form hybrids only following maturation and the concomitant recruitment of the *Plat* mRNA. Thus, the efficacy of this approach is compromised by the appropriate selection of the region of the mRNA to be targeted, and this can only be determined experimentally and not a priori.

Recently, RNA interference (RNAi), which employs double-stranded RNA (dsRNA), has been shown to ablate potently the targeted mRNA in a variety of species (Sanchez-Alvardado and Newmark, 1999; Fire et al., 1998; Kennerdell and Carthew, 1998; Li et al., 2000; Lohmann et al., 1999; Misquitta and Paterson, 1999; Ngo et al., 1998; Wargelius et al., 1999). The destruction of the targeted mRNA by dsRNA occurs prior to translation (Fire, 1999; Montgomery et al., 1998; Sharp, 1999; Zamore et al., 2000), and targets exon sequences; dsRNA directed against intron sequences is ineffective (Fire et al., 1998). Genetic approaches in *Caenorhabditis elegans* have identified genes with homology to eIF-2C, RNase D, and RNA-directed RNA polymerase (Ketting et al., 1999; Tabara et al., 1999; Smardon et al., 2000) that are involved in the RNAi-mediated pathway of mRNA degradation. Very recent studies suggest that a nuclease involved in the destruction of the targeted mRNA contains an essential RNA component containing approx. 25-nucleotide RNAs that are homologous to the dsRNA (Hammond et al., 2000). The processing of the dsRNA to these fragments does not require the presence of the targeted mRNA, and the targeted mRNA is cleaved only in the regions of identity to the dsRNA and at sites that are 21-23 nucleotides apart (Zamore et al., 2000).

We report here that dsRNA directed towards *Mos* and *Plat* mRNAs in mouse oocytes effectively results in the specific reduction of the targeted mRNA in both a time- and concentration-dependent manner. Moreover, dsRNA is more potent than either sense or antisense RNA. Targeting the *Mos* mRNA results in inhibiting the appearance of MAP kinase activity, as well as promoting parthenogenetic activation of the treated cells, and targeting *Plat* mRNA results in inhibiting production of tPA activity. Effective reduction of the *Mos* and *Plat* mRNA is observed with stoichiometric amounts of *Mos* and *Plat* dsRNA, respectively. While these studies were in progress, a paper appeared that has reported that oocytes injected with *Mos* dsRNA undergo egg activation, as evidenced by pronucleus formation (Wianny and Zernicka-Goetz, 2000).

MATERIALS AND METHODS

dsRNA preparation

For *Mos* amplification, a pair of primers was designed based on the cDNA sequence (Accession number J00372). The sequence of upstream *Mos* primer was 5′-CCATCAAGCAAGTAAACAAG-3′ and the downstream *Mos* primer was 5′-AGGGTGATTCCAAAAGA-GTA-3′. These primers generated a PCR product that was 535 bp in length and corresponded to the 3′ end of the coding region and the beginning of the 3′UTR. Likewise, for *Plat* amplification a pair of primers was designed based on the cDNA sequence (Accession number J03520). The sequence of the upstream *Plat* primer was 5′-CATGGGCAAGCGTTACACAG-3′ and the downstream *Plat* primer was 5′-CAGAGAAGAATGGAGACGAT-3′. These primers generated a PCR product that was 650 bp in length and corresponded to the middle part of the coding region.

To generate template for transcription in vitro, 5 μg of liver total RNA were reverse transcribed with Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions using oligo-dT as the primer. PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 4 minutes was followed by 36 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and the final cycle had an extended incubation at 72°C for 7 minutes followed by decrease to 4°C. All PCR reactions were performed in either a PE2400 or PE9600 PCR thermocycler.

Gel-purified primary PCR products were diluted 1:500 and re-amplified to produce specific templates to generate sense and antisense transcripts by transcription in vitro. To do this, primers were made that contained an SP6 promoter attached to the 5′ end of both the forward and reverse primers. Following PCR under the above conditions, the secondary PCR products were purified using a Nucleospin Extraction Kit (Clontech). The template (500-1000 ng) was then transcribed with SP6 RNA polymerase (Promega) in order to obtain the corresponding sense and antisense RNAs.

The in vitro transcription products were resolved following electrophoresis in 1.5% NuSieve LM agarose (FMC, Rockland, ME, USA) and the bands corresponding to the sense and antisense single-stranded RNA were purified according to the manufacturer's protocol. Equimolar amounts of sense and antisense RNA were then annealed in 1 mM Tris-HCl (pH 7.5), containing 1 mM EDTA, or in DEPC-treated water supplemented with 5% RNasin (Promega); similar results were obtained using either procedure. Typically, 2-4 μg of RNA in 30 μl were mixed and heated in 500 ml of boiling water for 1 minute. The sample, still in the water bath, was allowed to cool to room temperature over the course of several hours. The dsRNA was phenol extracted, ethanol precipitated, washed in 75% ethanol and then dissolved in water. Samples were stored in water at −80°C prior to use.

RNA isolation and RT-PCR

RNA was isolated from oocytes and prepared for RT-PCR as previously described (Temeles et al., 1994). In each case, 0.125 pg of rabbit β-globin mRNA/oocyte was added prior to RNA isolation. The globin mRNA serves as an internal standard for the efficiency of the RT-PCR reactions (Temeles et al., 1994). For each set of gene-specific primers the linear region of semi-log plots of the amount of PCR product as a function of cycle number was determined and a cycle number for each primer pair was selected that was in this linear range; the amount of PCR product under these conditions is proportional to the number of cells used (Manejwala et al., 1991). This method permits the comparison of relative changes in the abundance of a particular transcript (Ho et al., 1995; Latham et al., 1994; Temeles et al., 1994).

Following reverse transcription two oocyte equivalents were used as a template for each PCR reaction. PCR products were labeled with [α-^{32}P]dCTP (Amersham, 0.25 μCi per 50 μl reaction). PCR amplification conditions for both *Mos* and *Plat* were as follows: initial denaturation at 94°C for 2 minutes was followed by 28 (*Plat*) or 31 (*Mos*) cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by 4°C until the samples were removed. PCR amplification conditions for globin: initial denaturation at 94°C for 2 minutes was followed by 24 cycles of 94°C for 10 seconds and 62°C for 15 seconds followed by final 4°C. After PCR, the products were subjected to electrophoresis in an 8% polyacrylamide gel. The gel was dried under vacuum for 1 hour at 80°C, exposed in phosphorimager cassette for 4 to 24 hours and the signal was quantified using the Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Oocyte collection, microinjection and culture

Fully grown, germinal vesicle (GV)-intact oocytes were obtained from pregnant mare's serum gonadotropin (PMSG)-primed six-week-old female CF-1 mice (Harlan) and freed of attached cumulus cells, as previously described (Schultz et al., 1983). The collection medium was bicarbonate-free minimal essential medium (Earle's salt) supplemented with polyvinylpyrrolidone (3 mg/ml) and 25 mM Hepes, pH 7.3. Germinal vesicle breakdown was inhibited by including 0.2 mM 3-isobutyl-1-methyl-xanthine (IBMX). The oocytes were transferred into CZB medium (Chatot et al., 1989) containing 0.2 mM IBMX (CZB+IBMX) and cultured in an atmosphere of 5% CO_2 in air at 37°C. Oocytes were microinjected in bicarbonate-free CZB containing 10 mM Hepes and 0.2 mM IBMX with 5 pl of the corresponding solution; the injections were performed as previously described (Kurasawa et al., 1989). The concentration of the undiluted stock solution was 0.2 μg/μl and injection of 5 pl of either *Mos* or *Plat* dsRNA corresponds to 1.7×10^6 and 1.4×10^6 molecules, respectively. When single-stranded RNA was injected, it was diluted to a concentration such that injection of 5 pl corresponded to the same number of molecules as when dsRNA was injected. In experiments in which either enzyme activity or phenotype was assayed, microinjected oocytes were cultured in CZB+IBMX for 10 or 20 hours. They were then washed through ten drops of IBMX-free CZB and cultured in CZB until oocyte collection and lysis. In experiments in which mRNA levels were measured, the oocytes were kept in medium containing IBMX for 10, 20, or 40 hours until they were collected and processed for RNA isolation.

tPA assay

tPA activity was assayed by zymography of single oocytes. Immobilon-P (Millipore) was soaked in methanol for 1 minute and then rinsed four times with 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. The wet membrane was placed on Whatman paper soaked with this buffer and both were transferred to a 96-well dot blot apparatus (Milliblot Systems, Millipore) with the Immobilon-P facing upwards. A 96-well template was then placed on the stage and single oocytes were transferred in 1-2 μl of CZB medium to the middle of where the wells would form. The apparatus was completely assembled and the wet membrane with the oocytes was then exposed to vacuum suction for 2 minutes. The apparatus was then disassembled and the membrane was immediately applied on the detection gel; the detection gel, which contained 40 μg/ml of plasminogen (Fluka), was prepared as previously described (Vassalli et al., 1984). Zymograms were developed for 12-64 hours at 37°C, scanned with a black background and the lysed area was estimated using the ImageQuant software (Molecular Dynamics).

Histone H1 and MBP kinase assay

The activities of both histone H1 and myelin basic protein (MBP) kinases were determined in single eggs as follows: single eggs were transferred in 1.5 μl of culture medium into a tube containing 3.5 μl of double kinase lysis buffer (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 5 mM EGTA). The tubes were immediately frozen on dry ice and stored at −80°C until the assay was performed. The kinase reaction was initiated by the addition of 5 μl of double kinase buffer (24 mM *p*-nitrophenyl phosphate, 90 mM β-glycerophosphate, 24 mM $MgCl_2$, 24 mM EGTA, 0.2 mM EDTA, 4.6 mM sodium orthovanadate, 4 mM NaF, 1.6 mM dithiothreitol, 60 μg/ml aprotinin, 60 μg/ml leupeptin, 2 mg/ml polyvinyl alcohol, 2.2 μM protein kinase A inhibitor peptide (Sigma), 40 mM 3-(n-morpholino) propanesulfonic acid (MOPS), pH 7.2, 0.6 mM ATP, 2 mg/ml histone (type III-S, Sigma), 0.5 mg/ml MBP) with 500 μCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; Amersham). To determine the background level of phosphorylation for H1 and MBP, 5 μl of double kinase lysis buffer was added instead of the egg lysate. The reaction was conducted for 30 minutes at 30°C and terminated by the addition of 10 μl double-strength concentrated SDS-PAGE sample buffer (Laemmli, 1970) and boiling for 3 minutes. Following SDS-PAGE, the 15% gel was fixed in 10% acetic acid/30% methanol, dried and exposed to a phosphorimager screen for 16 to 24 hours. Scanning and quantification of the signal were performed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). For each experiment, the mean value of H1 or MBP kinase activities for oocytes microinjected with sense *Mos* RNA was arbitrarily set as 100% and the values obtained in the other groups of eggs were expressed relative to this amount.

RESULTS

Preparation of dsRNA

dsRNA for either *Mos* or *Plat* was prepared by hybridizing equimolar amounts of gel-purified, single-stranded sense and antisense transcripts that were generated by transcription in vitro of appropriate templates containing an SP6 promoter. In each case, the RNA was directed towards a coding portion of the transcript. Following hybridization, the single-stranded RNAs were essentially totally converted to dsRNA, as evidenced by the absence of any visible staining in the region of the gel that corresponded to single-stranded species (Fig. 1). The quantitative nature of hybridization permitted use of the dsRNA without any need for gel purification of the dsRNA species.

Effect of *Mos* and *Plat* dsRNA on *Mos* and *Plat* mRNA levels in mouse oocytes

Mos and *Plat* are two maternal mRNAs that are recruited during oocyte maturation (see Introduction). We selected to target the *Mos* mRNA since a *Mos* null oocyte has a defined phenotype, i.e., the oocyte matures to metaphase II, but rather than arresting at metaphase II, it undergoes spontaneous egg activation. In addition, it is possible to measure MAP kinase activity in a single oocyte; MAP kinase activity reflects the mobilization of *Mos* mRNA (see below). We also selected to target *Plat* mRNA, which like *Mos*, is a moderately abundant mRNA; it has been estimated that an oocyte contains approx. 10,000 transcripts each of *Mos* and *Plat* (Huarte et al., 1987;

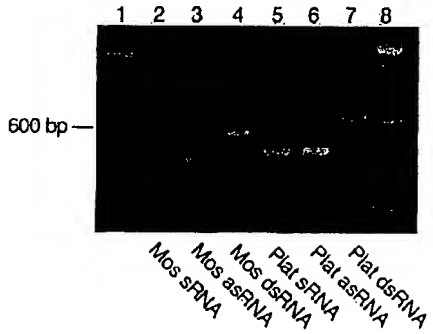

Fig. 1. Generation of *Mos* or *Plat* dsRNA. Sense or antisense *Mos* or *Plat* RNAs were produced by transcription in vitro and then gel purified. Equimolar concentrations of sense and antisense RNA were then hybridized and a portion of the reaction analyzed by electrophoresis. Shown is an ethidium bromide-stained gel demonstrating the quantitative formation of dsRNA. Lanes 1 and 8, 100 bp ladder.

Keshet et al., 1988). In addition, it is also possible to measure tPA activity in single oocytes.

Oocytes were injected with approx. 10^6 molecules of either *Plat* or *Mos* dsRNA that was directed towards the coding region of each transcript; this corresponds to approx. 10 nM final concentration (see Discussion). The oocytes were then cultured in medium containing IBMX to inhibit resumption of meiosis; a decrease in cAMP is associated with resumption of meiosis and including the membrane-permeable phosphodiesterase inhibitor IBMX in the medium prevents the decrease in cAMP and thus the resumption of meiosis (Schultz et al., 1983). Following culture, RNA was isolated and the relative amount of *Plat* and *Mos* transcripts were determined by a semi-quantitative RT-PCR assay that permits quantification of relative changes in transcript abundance. Prior to RNA isolation, a known amount of rabbit globin mRNA was added; this served as a control for RNA recovery, and for the efficiency of the RT-PCR (Temeles et al., 1994).

Oocytes injected with *Mos* dsRNA displayed a marked reduction in the amount of *Mos* transcript (approx. 80%), relative to water-injected or uninjected controls (Fig. 2, compare lane 2 with lanes 4 and 5). Likewise, oocytes injected with *Plat* dsRNA displayed an approx. 90% reduction in the amount of *Plat* transcript relative to the control (Fig. 2, lane 3). Specificity of this effect was demonstrated by the finding that *Mos* dsRNA did not reduce the abundance of *Plat* mRNA, and reciprocally, that *Plat* dsRNA did not reduce the abundance of *Mos* mRNA (Fig. 2). Results of these experiments indicated that the machinery for RNAi-mediated degradation of the targeted endogenous mRNA is present and functions in mouse oocytes.

Effect of *Mos* and *Plat* sense and antisense RNA on *Mos* and *Plat* mRNA levels in mouse oocytes

In other systems, antisense RNA can be ineffective. For example, injection of *C. elegans* with antisense RNA directed towards the *unc*-22 gene does not result in the mutant twitching phenotype, whereas dsRNA does (Fire et al., 1998).

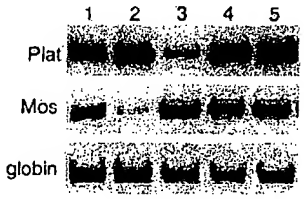

Fig. 2. Effect of *Mos* and *Plat* dsRNA on the relative abundance of *Mos* and *Plat* transcripts. Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR, as described in Materials and Methods. The intensity of the globin band permits comparison of the different lanes, as it normalizes for RNA recovery, and for the efficiency of the RT-PCR part of the assay. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0 hours; lane 2, relative amount of transcripts at t=20 hours in oocytes injected with *Mos* dsRNA; lane 3, relative amount of transcripts at t=20 hours in oocytes injected with *Plat* dsRNA; lane 4, relative amount of transcripts at t=20 hours in water-injected oocytes; lane 5, relative amount of transcripts at t=20 hours in uninjected oocytes.

Nevertheless, antisense RNA can be effective in degrading oocyte mRNAs (Strickland et al., 1988). Accordingly, we determined the effect of sense and antisense *Mos* and *Plat* RNA on targeting the cognate oocyte transcript.

Oocytes were injected with approx. 10^6 copies of either sense, antisense or dsRNA, and incubated for 20 hours in medium containing IBMX, before the RNA was isolated and transcript abundance determined. As anticipated, dsRNA directed towards either *Mos* or *Plat* mRNA resulted in the reduction of the targeted mRNA, whereas the untargeted transcript remained essentially intact (Fig. 3A). As also anticipated, injection of sense RNA resulted in little, if any, decrease in the abundance of either the targeted or nontargeted mRNA. Injection of either *Mos* or *Plat* antisense RNA, however, did result in a decrease in the targeted, but not in the nontargeted, mRNA (Fig. 3A). Little, if any decrease in the targeted mRNA was observed when the amount of injected *Mos* or *Plat* antisense RNA was decreased by 10-fold (Fig. 3B). In contrast; this amount of dsRNA was effective in decreasing the amount of the targeted mRNA (Fig. 3B), e.g., the *Mos* dsRNA resulted in an approx. 85% decrease in *Mos* mRNA, and *Plat* dsRNA resulted in an approx. 30% decrease in *Plat* mRNA. Results of these experiments suggest that dsRNA is more effective in targeting mRNAs than antisense RNA.

Concentration- and time-dependence of dsRNA directed towards *Mos* and *Plat* mRNAs

In the experiments described above, the oocytes were injected

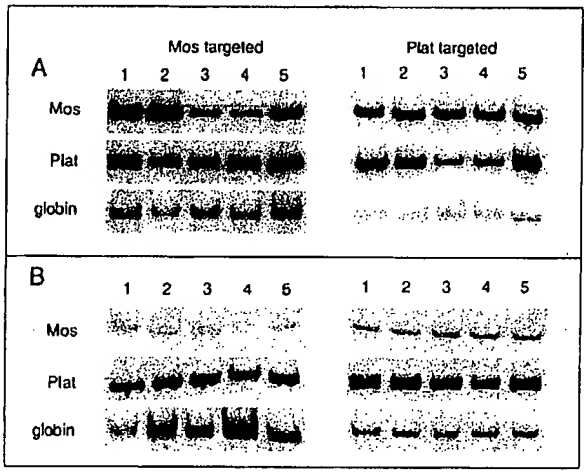

Fig. 3. Effect of *Mos* and *Plat* sense, antisense, and dsRNA on the relative abundance of *Mos* and *Plat* transcripts. (A) Oocytes were injected with approx. 10^6 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then cultured in the presence of IBMX for 20 hours. RNA was then isolated and the relative amount of *Mos* and *Plat* transcripts were determined by RT-PCR as described in the Materials and Methods. (B) Oocytes were injected with 10^5 molecules of either *Mos* or *Plat* sense, antisense or dsRNA and then processed as described in A. Lane 1, relative amount of transcripts present in water-injected oocytes; lane 2, relative amount of transcripts in sense RNA-injected oocytes; lane 3, relative amount of transcripts in antisense RNA-injected oocytes; lane 4, relative amount of transcripts in dsRNA-injected oocytes; lane 5, relative amount of transcripts in uninjected oocytes.

with approx. 10^6 molecules of dsRNA and cultured for 20 hours prior to determining the relative amount of targeted transcript. In order to determine further characteristics of the RNAi effect, the concentration- and time-dependence of this effect were determined. Oocytes were injected with 10^6, 10^4, or 10^2 molecules of either *Mos* or *Plat* dsRNA, and then incubated for 10, 20, or 40 hours prior to determining the relative abundance of the endogenous *Mos* and *Plat* transcripts (Fig. 4). For both *Mos* and *Plat* dsRNA-injected oocytes, the targeted message was destroyed in both a time- and concentration-dependent manner. In all cases, the nontargeted mRNA was not destroyed (data not shown).

Injection of 10^6 or 10^4 molecules of *Mos* dsRNA resulted in a substantial reduction in the amount of *Mos* mRNA, such that by 20 hours more than 75% of the mRNA was degraded; 10^2 molecules of injected *Mos* dsRNA had little, if any effect over the 40-hour timecourse. Although 10^6 molecules of injected *Plat* dsRNA also dramatically reduced the amount of *Plat* mRNA, the kinetics of *Plat* mRNA degradation were slower, when compared with those obtained for *Mos* dsRNA. In addition, 10^4 molecules of *Plat* dsRNA was not nearly effective as 10^4 molecules of *Mos* dsRNA. Similar to *Mos* dsRNA, the 100 molecules of injected *Plat* dsRNA was ineffective in reducing the amount of *Plat* mRNA.

Effect of Mos dsRNA on MAP kinase and p34^{cdc2}/cyclin B kinase activities

The experiments described above documented that both *Mos* and *Plat* dsRNA could result in the degradation of the targeted mRNA in a concentration- and time-dependent manner. We next demonstrated that the reduction of the targeted mRNA resulted in loss of formation of the encoded protein. The translation of *Mos* mRNA that initiates at the onset of oocyte maturation results in synthesis of MOS, which in turn activates MAP kinase kinase by phosphorylation (Gebauer and Richter, 1997; Sagata, 1997). MAP kinase kinase, which is a dual-specificity kinase, then phosphorylates MAP kinase on Thr183 and Tyr185 in the mammal, which in turn results in MAP kinase activation (Nishida and Gotoh, 1993). MAP kinase, which is a component of cytostatic factor (CSF) and is required to maintain metaphase II arrest, is frequently assayed by measuring the phoshorylation of MBP. Concomitant with germinal vesicle breakdown is the activation of p34^{cdc2}/cyclin B kinase (MPF) (Gebauer and Richter, 1997; Sagata, 1997), which is routinely assayed by phosphorylation of histone H1. In the mouse, MPF activation precedes MAP kinase activation by about 1-2 hours, and both activities reach maximal levels in the metaphase II-arrested egg (Verlhac et al., 1993). Following fertilization, MPF activity declines prior to MAP kinase activity (Moos et al., 1995; Verlhac et al., 1993).

Oocytes were injected with either *Mos* dsRNA, antisense RNA or sense RNA and cultured for 20 hours in IBMX-containing medium, then transferred to IBMX-free medium. The oocytes then matured to metaphase II, at which time both MAP and p34^{cdc2}/cyclin B kinase activities were assayed simultaneously in single eggs. As expected, sense RNA did not inhibit either kinase activity when compared with uninjected or water-injected eggs (data not shown). In contrast, both *Mos* antisense and dsRNA inhibited MAP kinase activity, although a greater degree of inhibition was observed with dsRNA (Fig. 5). This result was consistent with *Mos* dsRNA eliciting a greater decrease in *Mos* mRNA than *Mos* antisense RNA (Fig. 3). Although *Mos* antisense RNA did inhibit MAP kinase activity, the level of p34^{cdc2}/cyclin B kinase, i.e., histone H1 kinase, was reduced by only about 25% relative to control sense-injected or uninjected oocytes, while a 70% decrease was observed in the dsRNA-injected oocytes. This reduced amount of histone H1 kinase activity in the dsRNA-injected oocytes

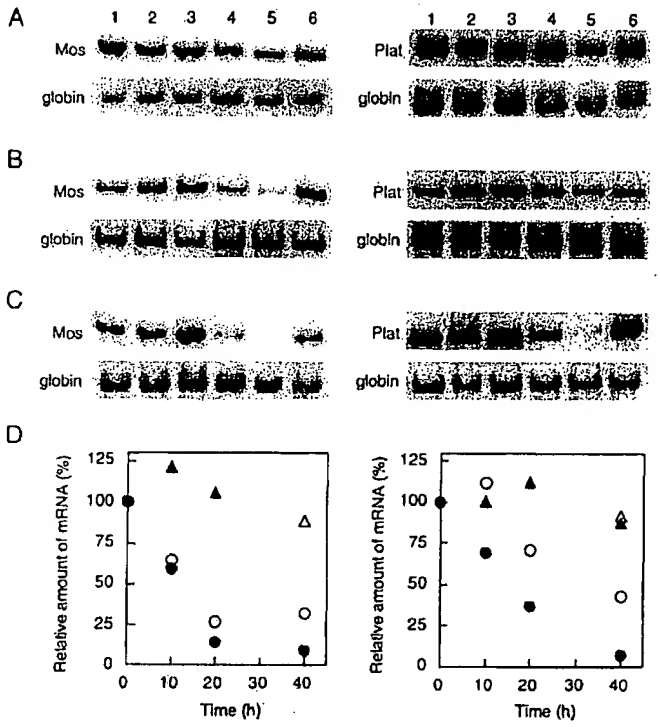

Fig. 4. Concentration- and time-dependence of *Mos* and *Plat* dsRNA-mediated reduction of the targeted mRNA. Oocytes were injected with 10^2 (lane 3), 10^4 (lane 4) or 10^6 (lane 5) molecules of either *Mos* or *Plat* dsRNA, and the relative abundance of either the *Mos* or *Plat* transcript was assayed after either 10 hours (A), 20 hours (B) or 40 hours (C) of culture in medium containing IBMX. Lane 1, relative amount of transcripts present in uninjected oocytes at t=0; lane 2, relative amount of transcripts in water-injected oocytes; lane 6, relative amount of transcripts in uninjected oocytes. (D) Quantification of the relative amount of *Mos* or *Plat* transcripts. The data are normalized to the amount present in the uninjected oocytes at the appropriate time following culture in IBMX-containing medium and all data are normalized to the globin signal, i.e., the ratio of the pixel volume of the transcript to that of the globin is set as 100%. (●), 10^6 molecules injected; (○), 10^4 molecules injected; (▲), 10^2 molecules injected; (△), the amount of *Plat* transcript present in oocytes injected with 10^6 molecules of *Mos* dsRNA or the amount of *Mos* transcript in oocytes injected with 10^6 molecules of *Plat* dsRNA. In order to keep the y-axis of similar scale, the value for oocytes injected with 10^2 molecules of *Mos* dsRNA and analyzed at 40 hours is not shown.

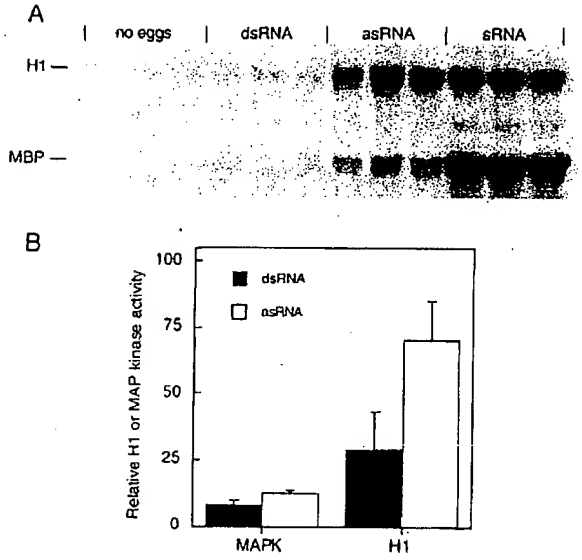

Fig. 5. Effect of *Mos* sense, antisense, and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^6 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds), and then incubated for 20 hours in IBMX-containing medium. The oocytes were then transferred to IBMX-free medium and allowed to mature to metaphase II (about 18 hours), at which time single oocytes were assayed for both MAP kinase activity and MPF activity using MBP and histone H1, respectively as substrates. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes (data not shown). The data are expressed as the mean±s.e.m. and represent a total of 15, 15 and 13 dsRNA-, asRNA- and sRNA-injected oocytes, respectively.

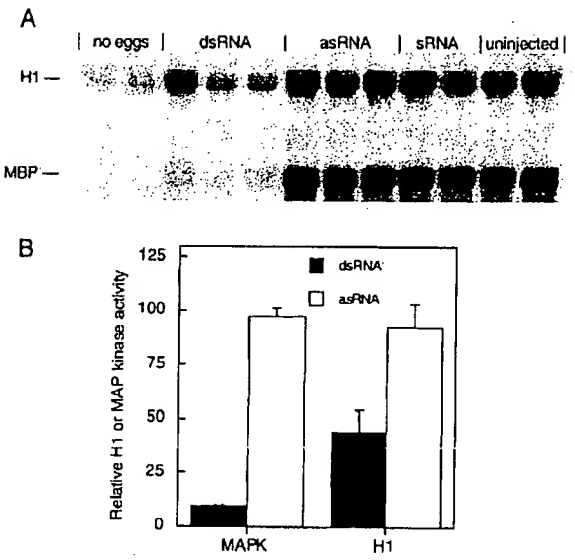

Fig. 6. Effect of *Mos* sense, antisense and dsRNA on MAP kinase and MPF activities. Oocytes were injected with 10^5 molecules of either *Mos* sense (s), antisense (as) or dsRNA (ds) and the experiment was then conducted as described in the legend to Fig. 5. (A) Region of autoradiogram showing where phosphorylated histone H1 and MBP migrate. (B) Relative amount of kinase activity. The data have been normalized to that present in oocytes injected with sense RNA and this value does not differ from uninjected oocytes. The data are expressed as the mean±s.e.m. and represent a total of 10, 10 and 6 dsRNA-, asRNA- and sRNA-injected oocytes.

was a consequence that in numerous cases these eggs underwent spontaneous egg activation, which results in a decrease in histone H1 kinase activity. In contrast, the antisense-injected oocytes never underwent egg activation.

Although *Mos* antisense RNA, which did result in a decrease in *Mos* mRNA, could inhibit MAP kinase activation, the results presented in Fig. 3 indicate that dsRNA is a more potent inhibitor than antisense RNA. Accordingly, oocytes were injected with 1/10 the amount of *Mos* sense, antisense, or dsRNA, cultured for 20 hours in IBMX-containing medium and then matured to metaphase II by transferring them to IBMX-free medium. The eggs were then assayed for both MAP and $p34^{cdc2}$/cyclin B kinase activities simultaneously in individual eggs. Whereas both *Mos* sense and antisense RNA did not inhibit the appearance of MAP kinase activity ($p34^{cdc2}$/cyclin B kinase activity was also high in these eggs), *Mos* dsRNA still elicited a dramatic inhibition in MAP kinase activity, and a corresponding decease in $p34^{cdc2}$/cyclin B kinase activity (Fig. 6). These results strengthen the conclusion that *Mos* dsRNA is more potent than *Mos* antisense RNA in promoting the reduction of the endogenous *Mos* mRNA.

Effect of Plat dsRNA on tPA activity

tPA is synthesized during oocyte maturation and its activity can be assayed in single oocytes by zymography (Huarte et al., 1985, 1987; Strickland et al., 1988). We observed only a single band (M_r 72,000) when metaphase II-arrested eggs were used; no activity was observed in GV-stage oocytes (data not shown). The presence of a single activity responsible for generating the lytic zone permitted analysis of tPA activity by simply spotting an oocyte/egg on a membrane, which was then overlaid with an agarose gel containing non-fat dry milk and plasminogen. The area of the lytic zone was linear as a function of time after a lag, which probably reflected the time to activate the zymogen cascade and degrade enough substrate to be visible to the eye (Fig. 7).

We assayed the effect of *Plat* sense, antisense and dsRNA on tPA activity in matured oocytes. Oocytes were injected with approx. 10^6 molecules of each RNA and cultured in IBMX-containing medium for 20 hours prior to initiating maturation by transfer to IBMX-free medium. Culture for 18 hours resulted in the production of metaphase II-arrested eggs that were then assayed for tPA activity. Injection of either antisense RNA or dsRNA resulted in a dramatic reduction in the amount of tPA activity, when compared with sense-injected oocytes (Fig. 8, black bars). The ability of *Plat* antisense RNA to inhibit the production of tPA activity following maturation is consistent with its ability to target the destruction of *Plat* mRNA (see Fig. 3A and Strickland et al., 1988), as well as its ability to inhibit translation of the *Plat* mRNA (Strickland et al., 1988). Nevertheless, injection of *Plat* sense RNA also modestly inhibited the production of tPA activity, although to

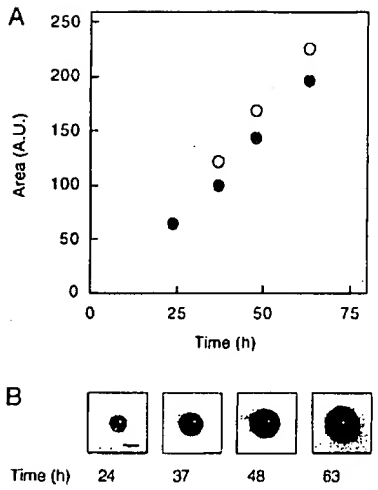

Fig. 7. Zymographic assay of tPA activity in single eggs. (A) Time-dependent increase in the area of the lytic zone of two eggs. (B) Photomicrographs of the lytic zone as a function of time of a single egg. Scale bar: 3 mm.

a lesser extent than either *Plat* antisense or dsRNA (Fig. 8, black bars). We observed that *Mos* sense RNA, which doesn't target the *Plat* mRNA, also resulted in a 50% decrease in tPA activity but had no inhibitory effect on the activation of MAP kinase (data not shown). The molecular basis underlying this inhibitory effect of sense RNA on the generation of tPA activity remains unresolved.

The results presented in Fig. 3 indicated that *Plat* dsRNA was more potent than *Plat* antisense RNA in targeting the reduction of endogenous *Plat* mRNA. As expected, a ten-fold dilution of *Plat* antisense RNA resulted in levels of tPA activity

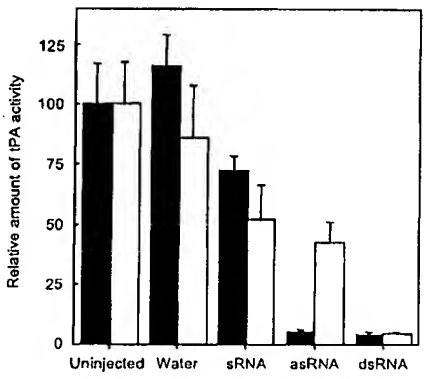

Fig. 8. Effect of *Plat* sense (s), antisense (as) and dsRNA (ds) on the appearance of tPA activity following oocyte maturation. Black bars, injection of approx. 10^6 molecules of RNA; white bars, injection of approx. 10^5 molecules of RNA. The value obtained in the uninjected oocytes was taken as 100% and the other samples are expressed relative to this amount. The data are expressed as the mean±s.e.m. and typically 5-12 eggs were assayed. The experiment was performed three times and similar results were obtained in each case; a representative experiment is shown.

similar to that of sense-injected oocytes, whereas injection of *Plat* dsRNA still promoted a dramatic inhibition (Fig. 8, white bars). Similar to the results obtained with *Mos* dsRNA, these results confirmed that *Plat* dsRNA was more potent than *Plat* antisense RNA in promoting the reduction of the endogenous *Plat* mRNA.

DISCUSSION

We have demonstrated that RNAi is an effective and efficient method to inhibit the translation of maternal mRNAs that are recruited during oocyte maturation. The reduction of the targeted mRNA, namely *Mos* and *Plat*, is specific, i.e., a nontargeted mRNA is not destroyed, and is both time- and concentration-dependent; mRNA levels can be decreased by up to 90%. In addition, dsRNA is more effective than antisense RNA. The reduction of *Mos* mRNA led to a failure in MAP kinase activation that normally accompanies oocyte maturation. A consequence of this failure is that metaphase II arrest was not maintained and that the eggs underwent parthenogenetic activation with the concomitant decrease in H1 kinase activity. Likewise, reduction of the endogenous *Plat* mRNA inhibited the production of tPA following oocyte maturation. A recent report also found that injection of *Mos* dsRNA results in parthenogenetic activation of mouse eggs (Wianny and Zernicka-Goetz, 2000); it was not shown in that study, however, that the *Mos* RNA is selectively degraded and that MAP kinase failed to activate. In the mouse, RNAi, which entails microinjection of the dsRNA, should prove far superior to antisense approaches that have been used in the past, but with variable success. It should be noted that culture of oocytes in medium containing either *Plat* or *Mos* dsRNA (2 μg/μl) does not reduce the amount of the targeted mRNA (P. S., P. S. and R. M. S., unpublished). Thus, in contrast to lower species such as *C. elegans* (Fire et al., 1998) and planaria (Sanchez-Alvarado and Newmark, 1999), in which injection of the dsRNA into the animal results in the reduction of the targeted mRNA, mouse oocytes apparently lack this uptake mechanism or, if it is present, it is very inefficient.

About 1.5×10^6 molecules of either *Plat* or *Mos* dsRNA were injected when undiluted dsRNA was used. This corresponds to an intracellular concentration of 10 nM, as the volume of an oocyte is approx. 250 pl. This concentration is similar to that required to ablate *frizzled* function in *Drosophila* embryos (Kennerdell and Carthew, 1998). In those experiments approx. 0.2 fmole of dsRNA was injected into syncytial blastoderm embryos whose volume is approx. 7.3 nl and this corresponds to approx. 25 nM. Concentrations of 10 nM dsRNA are effective in an in vitro system that supports the destruction of the targeted mRNA (Tuschl et al., 1999). Significant reduction of both *Mos* and *Plat* mRNAs are also observed when only 10,000 molecules of *Mos* or *Plat* dsRNA are injected. As the oocyte contains about 10,000 each of these transcripts (Huarte et al., 1987; Keshet et al., 1988), the reduction of the endogenous mRNA appears to be very efficient. A catalytic mechanism is possible, as in other systems the number of dsRNA molecules per cell is likely to be less than the number of endogenous transcripts. For example, in *C. elegans*, injection of 60,000 *unc*-22 dsRNA into adult animals results in the twitching phenotype in approx. 100 progeny (Fire et al.,

1998). *unc*-22 expression commences when the embryos contain about 500 cells, by which time the injected dsRNA would be diluted to only a few molecules per cell. Alternatively, the recent finding that an RNA-directed RNA polymerase is implicated in RNAi (Smardon et al., 2000) could provide an amplification mechanism that accounts for the efficacy of stoichiometric or substoichiometric amounts of dsRNA to promote the efficient reduction of the targeted mRNA.

When approx. 10^6 or 10^4 molecules of either *Mos* or *Plat* dsRNA are injected, the kinetics of *Plat* mRNA degradation are slower than that for *Mos* mRNA. As it has been estimated that oocytes contain approx. 10 000 transcripts of each of these mRNAs, the difference in kinetics of mRNA degradation may reflect that the *Mos* mRNA is more accessible to be targeted for destruction. It should be borne in mind, however, that estimate of the number of transcripts is relatively crude, and hence the difference in kinetics of mRNA degradation may reflect differences in transcript abundance, i.e., there is less *Mos* mRNA than *Plat* mRNA.

Both *Mos* and *Plat* antisense RNA are also effective in reducing the amount of endogenous *Mos* and *Plat* mRNA, respectively. Nevertheless, on a molar basis, the antisense RNA is not as effective as dsRNA. For example, *Mos* dsRNA more effectively inhibits the activation of MAP kinase when compared with *Mos* antisense RNA; parthenogenetic activation and the concomitant reduction in histone H1 kinase activity are only observed in *Mos* dsRNA-injected eggs, and not in *Mos* antisense RNA-injected eggs. This suggests in turn that MAP kinase activity must be reduced below a threshold level at which MAP kinase activity is almost absent, in order to make the eggs exit metaphase II arrest and enter interphase. Moreover, when the amount of injected *Mos* dsRNA and antisense RNA are reduced 10-fold, *Mos* dsRNA is still highly effective in inhibiting the increase in MAP kinase activity whereas *Mos* antisense RNA is essentially ineffective. Thus, *Mos* dsRNA is more efficient than *Mos* antisense RNA. A similar situation is also found with *Plat* antisense and dsRNA. Injection of 10^6 molecules of *Plat* antisense or dsRNA results in both destroying the *Plat* mRNA and inhibiting the increase in tPA activity that accompanies oocyte maturation. In contrast, injection of 10^5 molecules of *Plat* antisense RNA results in little reduction of the endogenous mRNA and little inhibition in the increase in tPA activity, while *Plat* dsRNA still results in the reduction of the endogenous mRNA and inhibition of the appearance of tPA activity.

The increased potency of dsRNA when compared with antisense RNA could, in principle, reflect differences in their stability, i.e., dsRNA is more stable than antisense RNA. This possibility is minimized by the observation that in a *Drosophila* cell lysate that supports RNAi-mediated mRNA destruction both capped antisense and capped dsRNA are stable but only the capped dsRNA is active (Tuschl et al., 1999). Moreover, results of recent experiments suggest that processing the dsRNA to discrete 20-25 nucleotide fragments is part of the mechanism that leads to destruction of the targeted mRNA (Hammond et al., 2000; Zamore et al., 2000). In fact, asRNA can give rise in an in vitro system to small amounts of stable 20-25 nucleotide fragments (Zamore et al., 2000). This could account for the activity, albeit reduced, of antisense RNA, relative to dsRNA.

dsRNAs in mammalian cells typically activate protein kinase PKR that phosphorylates and inactivates eIF2a (Fire, 1999). The ensuing inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles. Mouse oocytes, however, clearly lack this response, as oocyte maturation beyond germinal vesicle breakdown requires protein synthesis (Wassarman et al., 1976), which probably reflects a requirement for cyclin B synthesis, and the oocytes injected with dsRNAs resume meiosis and mature to metaphase II. Preimplantation mouse embryos also lack the response, as embryos injected with dsRNAs develop to the blastocyst stage (Wianny and Zernicka-Goetz, 2000). When the embryo acquires this response is unknown. It is not known if oocytes and preimplantation embryos contain PKR activity, which could account for the lack of the response. The lack of this response, however, cannot be attributed to a deficiency in the cell death machinery, because both oocytes (Perez et al., 1999) and preimplantation embryos (Brison and Schultz, 1997; Handyside and Hunter, 1986; Pierce et al., 1989; Weil et al., 1996) can undergo apoptosis.

The lack of this response to dsRNA may confer a selective advantage by minimizing reproductive wastage. Both oocytes and preimplantation embryos are exposed to viruses. Viral exposure throughout the lifespan of the female could deplete the pool of oocytes and compromise her reproductive capacity, because oocytes do not proliferate. The preimplantation embryo is also susceptible to viral infection from viruses present in the female reproductive tract. Infection of an early cleavage stage preimplantation embryo that results in blastomere death could result in a blastocyst containing an insufficient number of inner cell mass cells, and hence be incapable of development to term.

RNAi clearly offers several advantages to the current methods that employ generation of null mutants by homologous recombination, which requires (1) generating a suitable targeting construct, (2) selecting homologous recombination events in ES cells, (3) injecting blastocysts with these ES cells, and (4) establishing pure breeding lines from the chimeric offspring. The RNAi response will also likely be far more efficient and consistent than the antisense RNA approach that has been used with very inconsistent results in the mouse oocyte and embryo. Moreover, hypomorph phenotypes may become manifest, as RNAi does not appear to result in the total ablation of the targeted mRNA. Such hypomorph phenotypes may be as informative (or more informative) than the corresponding null mutation by providing novel insights into the presence of thresholds and/or the function of a gene. For example, as described above, a critical amount of MOS activity appears required for the development of a level of MAP kinase activity that is sufficient to maintain metaphase II arrest, a result consistent with a recently proposed switch mechanism for MAP kinase activation, as well as other cellular switches (Ferrell, 1999). In addition, modest changes in the levels of *Oct4* (*Pou5f1* – Mouse Genome Informatics) expression may also function as a developmental switch by regulating the fate of embryonic stem cells, e.g., high levels lead to differentiation into primitive endoderm and mesoderm, intermediate levels lead to pluripotent stem cells and reduced levels result in trophectoderm (Niwa et al., 2000).

As more dormant maternal mRNAs are identified, RNAi will be a valuable tool with which to study their function in oocyte maturation, fertilization and egg activation, and development. Moreover, the method can also be used to study the function of genes that are expressed in the early embryo, since dsRNA can inhibit the function of zygotically expressed genes (Wianny and Zernicka-Goetz, 2000; P. S., P. S. and R. M. S., unpublished). Whether dsRNA can also lead to DNA methylation of the targeted gene and result in long-term repression of transcription, as apparently occurs in plants (Wassenegger et al., 1994), is unknown.

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EXHIBIT 8

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Patent application of Alan Gewirtz	:	
	:	Group Art Unit: 1635
Serial No.: 09/993,183	:	
Filed: November 14, 2001	:	Examiner: Jon B. Ashen
For: POST-TRANSCRIPTIONAL GENE SILENCING BY RNAi IN MAMMALIAN CELLS	:	Conf. No. 6995

DECLARATION OF DR. ALAN M. GEWIRTZ under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Alan M. Gewirtz, declare that:

1. I am the inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 09/993,183, claiming for the first time that it is possible to disrupt gene expression at the mRNA level in a target gene in a *human* cell by providing to that human cell, small interfering double stranded RNA (dsRNA) guide sequences that are homologous to a portion of the target gene, such that RNA interference (RNAi) of the target gene is induced, and I have provided evidence demonstrating that effect in several human cell lines.

2. To facilitate submission of this Declaration, I have not attached a copy of my *curriculum vitae*. Should my c.v. become required, I will produce it upon request. Briefly, however, my career has been devoted to studies of normal and malignant human hematopoiesis, translational strategies for silencing gene expression, and generally, to ways of translating basic scientific discoveries into clinically useful therapies for patients with disorders of blood cell formation. I am currently Professor of Internal Medicine and Pathology-Laboratory Medicine at the University of Pennsylvania School of Medicine, Division of Hematology/Oncology, and hold a number of faculty appointments at the University, including Leader of the Hematologic Malignancies Program in the Cancer Center,

and Member in the Cell and Molecular Biology, and the Pharmacology Graduate Groups. I hold an M.D. degree and an M.A. in microbiology from the State University of New York at Buffalo. In 1976, I began an Internship and Residency at Mt. Sinai Hospital in New York City which I completed in 1979. I then accepted Fellowships in both Hematology and Oncology at Yale University, School of Medicine, in New Haven, Connecticut which were completed in 1982. I am Board Certified in both Specialties. Upon completion of my fellowship training I received an Instructor appointment in the Dept. of Medicine at Yale which I resigned after one year to accept a position as Assistant Professor of Medicine and Thrombosis Research at Temple University in Philadelphia. I remained at Temple University through 1990 at which time I moved to the University of Pennsylvania to assume the position of Associate Professor (with academic tenure) in the Departments of Pathology and Medicine. I have remained at Penn ever since. Presently, I am hold the titles of Professor of Medicine, and Pathology and Laboratory Medicine, and Leader of the Hematologic Malignances Program at the University of Pennsylvania Cancer Center. I am also a full Member of the Leonard and Madelyn Abramson Family Cancer Research Institute. I was elected to the American Society of Clinical Investigation in 1990, and I have been honored with several awards, including the Scientific Achievement Award from the American Cancer Society; the Doris Duke Distinguished Clinical Scientist Award for Excellence in Bench to Bedside Research, William Osler Award for Patient Oriented Research (Univ. of PA); and recently, I was installed as the first C. Willard Robinson Professor of Hematology/Oncology at the University of Pennsylvania School of Medicine. I have served on numerous ad hoc NIH and foreign government (Canada, Switzerland, United Kingdom) review panels and served full terms on the NIH Experimental Therapeutics Study Section-I, and the Hematology Study Section of the Veterans Administration. I am a member of several editorial boards for specialty journals concerned with human stem cells, hematopoiesis and gene therapy, including Nucleic Acids Research and the Journal of Clinical Oncology. In addition, I recently chaired the Medical/Scientific Advisory Committee of the Leukemia and Lymphoma Society and I currently serve on its newly formed Board of Directors.

3. I have read all of the communications from the U.S. Patent and Trademark Office (PTO) relating to the above-identified application, and I have participated in the Response process for each Office Action. I was also an active participant at the in-person Examiner's

Interview for this application on July 18, 2005. Consequently, having talked to Examiners Ashen and Wang at length on the subject of my invention and having discussed with them their comments made in the Office Action dated May 23, 2005, I am well qualified to address their concerns and explain why my discovery of the inhibitory effect of RNAi on a target gene in a *human* cell, based upon actual testing in my laboratory initiated prior to Fall 2000, was not, and could not have been, anticipated, predicted or suggested by any prior teachings in the art.

4. Claims 1, 2, 5, 7-9, 11, 21-27 are pending in the application. Claims 1 and 22 are independent claims, and all other pending claims are directly or indirectly dependent upon either claim 1 or claim 22. As presently amended, claims 1 and 22 read as follows:

> 1. A method for disrupting target gene expression at the mRNA level in a human cell, wherein the method comprises initiating RNA interference (RNAi) *in vitro* by exposing the cell to a double stranded RNA (dsRNA) homologous to the target gene, wherein the dsRNA consists essentially of two complementary linearized strands of RNA, the transcription of each is independently controlled to generate paired RNAs of defined length.
>
> 22. A method for disrupting target gene expression *in vitro* at the mRNA level in a human cell, wherein the method comprises providing small interfering RNA guide sequences which are homologous to a portion of the target gene, such that RNAi of the target gene is induced.

5. It is my understanding that according to the Examiner in the Office Action dated May 23, 2005, paragraph 8, the pending claims are rejected under 35 U.S.C. 102(e) as anticipated by Fire *et al.* (US Pat. No. 6,506,559) because the Fire patent discloses "a method for inhibiting expression of a target gene using double stranded RNA to induce RNAi in a cell in vitro (col. 26, claim 1) wherein the cell is from an animal (col. 26, claim 6)." In making this argument, the Examiner states that "Fire *et al.* disclose that the cell with the target gene may be derived from or contained in any organism (col. 8, line 13-14) and that examples of vertebrate animals include mammals and human (col. 8, lines 35-37)."

6. In arguments of record, I have, through my counsel, argued that such a rejection is improper and incorrect. In fact, at the time of the '559 invention by Fire *et al.* (U.S. effective filing date claimed was December 23,1997, actual US filing date was December 18, 1998), he and his co-inventors knew that the claimed method of using dsRNA inhibited expression only of a target gene in cells of an invertebrate embryo, *C. elegans*. As has been previously pointed out in the present record, in later publications Fire acknowledged that he did not actually believe that

his claimed method would actually work in a human cell. Others agreed that, at the time, there were many reasons for one skilled in the art to doubt that Fire's method could or would be effective, as claimed in the '559 patent, in a human cell. To suggest that Fire's specification, containing data showing *only* the effect of dsRNA in embryonic invertebrate cells, would suggest to one of ordinary skill at that time, that the method also taught how to inhibit expression of a target gene in a human cell, is to read far more into the '559 patent than was actually in the inventor's possession at the time of his invention or that he could have known. This is especially true given Fire's own subsequently published words questioning the effectiveness of his invention in a mammalian cell.

7. Fire's claim 1 is, in fact, silent on the cells for which his method is effective, although dependent claims specify an "animal" - without saying vertebrate or non-vertebrate, and subsequently clarifies that the cells are from a plant, invertebrate and nematode. While the '559 specification states at col. 8, line 16, that "the animal *may* be a vertebrate or invertebrate" (emphasis added), no other statement teaching or example in the entire application, other than at column 8, addresses any dsRNA response in a cell taken from a "vertebrate," or specifically from a human. Thus, the boilerplate lists included in the patent specification were merely wishful-thinking by Fire when the '559 specification was drafted. Evidence shows that Fire had no idea whether the system would or could operate in any cell other than an embryonic *C. elegans* cell, and in fact, there was strong evidence at the time to show why it would not operate in vertebrate cells.

8. Prior to my own filing date of November 2000, data were never presented by Fire, in the '559 specification or elsewhere, showing the effect of dsRNA on a vertebrate cell. And based upon the inventor's subsequent publications, he never tested to ascertain whether dsRNA could inhibit expression in a target gene in a vertebrate cell. While confirmation that the Fire '559 invention would actually function in a mammalian cell may not always be required of an inventor under U.S. Patent Law, some level of proof certainly would have been expected by one of ordinary skill in this art before accepting such a claim – since it was contrary to the recognized state of the art at the time of the Fire invention in 1998. At that time, and for several years thereafter, the expected response of post-embryonic mammalian cells, was known in the art to be very different from the operation of the '559 invention in an embryonic *C. elegans* cell. Yet the inventor only tested his invention using invertebrate cells. Fire certainly never made any

statement in the '559 specification or later, that would suggest that he believed that his invention would actually work in a vertebrate, mammalian or human cell. He simply included everything possible in his long boilerplate paragraphs – and even then preceded the list with a "may be." Such lists certainly would not have led one of ordinary skill in 1997 or 1998 to attempt to practice the '559 invention in a vertebrate cell, because dsRNA inhibition of a target gene in a vertebrate cell was contrary to the response expected in such cells at the time, particularly when read in the context of published conflicting statements by the inventor himself about the effectiveness of the invention in mammalian cells. In fact, in the '559 specification, Fire failed to even identify the recognized and substantial defense mechanisms that one would have expected in a mammalian cell. Yet these defenses would have made the operation of dsRNA very different in such a mammalian cell, as compared to the '559 disclosed method of operation of dsRNA in an invertebrate embryonic cell.

9. Fire authored at least two papers (cited below) well after the effective filing date of the '559 patent, in which the very speculative nature of RNAi in mammalian cells, such as mice and humans, was made clear. Both have been previously provided to the Office.

10. In Montgomery and Fire, *TIG* 14:255-258 (1998), the authors discussed the inhibitory effect of dsRNA in embryonic *C. elegans* cells, and then ended the article with a discussion of their expected response of mammalian cells to dsRNA. Specifically, they note that a mammalian cell exposed to dsRNA "unleashes a vehement but somewhat non-specific response leading to general translational arrest" (page 258 sentence bridging columns 1 and 2). This phenomenon found in mammalian cells, refers to the effect of a cellular kinase known as PKR (protein kinase RNA-activated) which is induced by interferon and activated by RNA. The PKR response is one of several mammalian intracellular defense mechanisms, designed to inhibit viral infection, which operates by shutting down translation in a cell in the presence of long dsRNA, and ultimately leads to cell death by apoptosis. Fire and his co-author, thus speculated that "any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or a controlled level of dsRNA that was incapable of activating PKR," but how to accomplish either proposal was not mentioned in either the publication or in the earlier-filed patent.

11. Fire *et al.* offered neither their own teachings, nor pointed to any method known at the time for achieving such a lapse in the PKR response in post-embryonic, PKR-proficient, mammalian cells. Nor did they explain any reason why such expected defense mechanisms would not operate against the introduction of dsRNA into mammalian cells. In fact, the authors further indicated that, while there were suggestions in the literature of RNA-mediated interference in gene expression control, it remained unknown whether the RNA that induced such interference was single-stranded or double-stranded. See page 258, first full paragraph, second column. These statements clearly indicate that at that time in 1998, following the 1997 effective filing date of the application for the '559 patent, Fire himself recognized that any attempt to practice his invention as claimed in a mammalian cell, would be expected to result in a "vehement" PKR response that would render his method ineffective in any PKR-proficient mammalian cell, yet he had no idea how to overcome such a problem. Therefore, based upon the inventors' own statements, one skilled in the art would not have attempted to practice the '559 invention in a mammalian cell, since the recognized art at the time was that the PKR response and other mammalian defenses would violently block the effectiveness of dsRNA. As a result, based upon the '559 patent's teaching *only* of the response in embryonic invertebrate cells, in light of the mammalian defense mechanisms recognized in 1997 and 1998, such an individual would certainly not have expected dsRNA to induce RNA interference in a human cell, particularly since no data or explanation was provided by Fire regarding mammalian cell responses to dsRNA.

12. In *Trends Genet.* 15:358-363 (1999), Fire reiterated his concerns that his invention, as practiced in *C. elegans* embryonic cells, could not be practiced in a mammalian cell. On pp. 362-363 under "Real-world applications: what about us?" Fire states that "one *could* certainly *hope* that RNA-triggered silencing would exist in vertebrates" (emphasis added). Such a statement made at least a year after the 1997 effective filing date or 1998 actual filing date of the '559 patent, clearly shows that, at the filing date of the application for the '559 patent, Fire was not in possession of such information. As a result, Fire then continued in the 1999 paper that

> ". . . the simple protocols used for invertebrates and plant systems *are unlikely to be effective* [in vertebrates]" (emphasis added).

13. In fact, Fire had no data or reason to believe that the expected PKR response or other intracellular defenses to foreign dsRNA could be overcome in a vertebrate cell. Accordingly, at

the filing date of the '559 patent (1998), one skilled in the art, *i.e.*, Fire himself, did not believe his claimed methods would work in mammalian cells. By 1999, Fire stated that he still did not believe that his invention could be applied to mammalian cells without substantial additional research, *i.e.*, it was "unlikely to be effective." Consequently, it would certainly be accurate to say that such statements by Fire actually would have discouraged one of ordinary skill in the art from attempting to practice the '559 invention in vertebrate cells. Certainly, the 1999 publication taught one away from attempting to use dsRNA – in accordance with the method claimed in the '559 patent - in vertebrate or human cells.

14. A review of more recent literature confirms that as recently as 2001, neither Fire, nor anyone else, knew what response to expect from the introduction of dsRNA to a human cell. This was 4 years AFTER the effective filing date of the '559 patent application, and well after publication of the cited Fire manuscripts. However, what is clear is that the art did not believe that the Fire method would be effective in mammalian cells. Paddison *et al.*, *Proc. Nat'l Acad. Sci.* 99(3):1443-1448 (Feb. 5, 2002) (attached to show the subsequent state of the art) was presented and discussed at length with the Examiners in the July in-person interview. In the 2002 paper, Paddison *et al.* state (first and second columns, page 1443) that:

> It has become clear that ds-RNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans. . .
> In several systems, dsRNA-induced silencing has been harnessed as a powerful tool for the analysis of gene function. Particularly in *Caenorhabditis elegans*, RNAi has emerged as the standard protocol for quickly assessing the consequences of inhibiting gene function. . . . In *Drosophila*, the first evidence of dsRNA-induced silencing came from the study of embryos, and insects. (citations omitted)

Standing alone, the preceding section would, like an unduly broad interpretation of the Fire patent, suggest that the teaching in *C. elegans* was the accepted standard for dsRNA to inhibit target gene function in *all* cells. But, in fact, when one continues to read Paddison *et al.* on page 1443, the authors clearly distinguish the art-recognized response of a vertebrate cell, from that of an invertebrate cell:

> Despite its utility in diverse systems, harnessing RNA to study gene function in mammals seemed potentially problematic. Indeed, mammals have evolved robust systems for responding to dsRNAs . . .

Discussion then continues regarding mammalian intracellular defense mechanisms that are not found in the invertebrate cells that were tested by Fire in the '559 patent.

15. Then, in the Discussion section (first column, page 1448), Paddison *et al.* clearly state that:

> The first indications that this response [to dsRNA] might also extend to mammals came from the observation that injections of dsRNAs into early mouse embryos induced sequence-specific silencing (23,24). Recent work by Tuschl and colleagues (5) had shown that siRNA can induce silencing in numerous mammalian cell lines . . . .

16. While the point of the discussion by Paddison et al. was directed to the transient nature of the gene silencing achieved by the early examination of the effect of dsRNA on mammals, several points are made clear. First, and foremost, the *first* researchers to publish results of dsRNA in a mammalian cell were Wianny *et al., Nat. Cell Biol.* 2:70-75 (2000) (cited reference 23) and Svoboda *et al., Development (Cambridge U.K.)* 127:4147-4156 (2000) (cited reference 24). Wianny *et al.* state in first column, page 71 that:

> So far *there has been no report that RNAi can be used in mammals.* Moreover, there are several indications of potential limitations to its [dsRNA] function in this group of animals [mammals]. Principal among these is that the accumulation of very small amounts of dsRNA in mammalian cells following viral infection results in the interferon response, which leads to an overall block to translation and the onset of apoptosis. *Such considerations have discouraged investigators from using RNAi in mammals.* Emphasis added, cited references omitted.

17. Evidently, Fire's published work in invertebrate cells and the '559 patent, as well as his subsequent publications, are not even considered by other authors to be relevant to a discovery of the effect of dsRNA in mammalian cells. Moreover, the early work by Wianny *et al.* and by Svoboda *et al.*, each of which has been discussed at length in the recorded prosecution history of the present invention, relate to embryonic mammalian cells in which the defense mechanisms have not yet developed. Only the work by Tuschl *et al.* (cited as reference 5, Elbashir et al., Nature (London) 411:494-498 (2001)) demonstrated the effect of dsRNA in mature mammalian cell lines – and that work was not reported until at least 5 months AFTER the effective filing date of my invention in November 2000.

18. The Examiner in the most recent Office Action, dated May 23, 2005, found our arguments to be unpersuasive. While acknowledging that the above-cited references recognized "potential difficulties in the application of dsRNA into mammalian cells," the Examiner found that the art also provided "a reasonable means of overcoming said difficulties; *e.g.*; by using a controlled level of dsRNA." However, I must strongly express my disagreement with such an argument for at least three reasons.

19. First, Fire made no mention in the '559 patent of the difficulties to be overcome in dealing with the intracellular defenses of mammalian cells. In fact, no difference was noted at all in the '559 patent between the expected response in a vertebrate cell, as compared with the data presented in an embryonic invertebrate cell – despite the fact that the state of the art at the time was well aware of the significant differences between the two, and of the defenses present in a viable mammalian cell. One could speculate that perhaps Fire was not aware of the differences. However, in a publication in 1998, he acknowledged that one would expect to encounter "vehement" defenses if dsRNA were introduced into a mammalian cell, and that such defenses would be expected to lead to translational arrest. In other words, Fire knew of the problems to be encountered, and expected that they would, indeed, probably block the silencing effect of the introduced dsRNA in a mammalian cell, yet he failed to identify the potential problem, nor did he offer any solution to the problem in the '559 specification. This is because neither he, nor anyone else, knew of a solution at the time of the filing date of the '559 invention. At that time, Fire simply was not in possession of an invention beyond the use of dsRNA in an embryonic invertebrate cell that would not encounter such intracellular defenses.

20. Second, while years after the filing date of the '559 patent, *others* clearly recognized problems associated with any attempt to apply the Fire methods (enabled only in embryonic invertebrate cells) to mammalian cells, and may have proposed solutions to the expected intracellular defenses - that is irrelevant to the rejection of my invention over the Fire patent. The fact is that Fire later acknowledged that use of his invention in vertebrate cells would encounter "vehement" defenses that would block the silencing of the target gene, yet he failed to address that anticipated problem in the '559 specification. The state of the art in 1997 and 1998 clearly showed that one of ordinary skill in the art would not have expected to use, or even tried to use, the '559 invention in a mammalian cell without being prepared to deal with the expected intracellular defenses. Fire recognized the problem, but offered no solution at the time of his

invention or later – presumably because he knew of no solution that would permit the use of his invention in mammalian cells. It would be improper and unfair to now credit Fire with an impermissibly broad interpretation of his claimed invention, extending far beyond what he enabled or possessed, to find my invention unpatentable – even though I accomplished what no one before me had done, or believed could be done – that is, to introduce dsRNA into a human cell and demonstrate the resulting disrupted expression of a target gene.

21. Third, the Examiners have said that my invention, a method for disrupting target gene expression at the mRNA level in a *human* cell, as defined in my above-identified claims, is unpatentable over the Fire invention because the '559 patent anticipated and taught every element of my invention before I did. If that were the case, then why was the work of Wianny *et al.* and Svoboda *et al.* considered "pioneering" in 2002 when others discussed the state of the art? Those researchers only dealt with embryonic mammalian cells in which the defense mechanisms have not yet developed, yet their work was considered a break-through because it addressed a problem that had not been previously solved in the art. It is clear that no one in 2002 recognized Fire's work in 1997 or 1998 as sufficient to permit one of ordinary skill in the art to practice his claimed invention in a mammalian cell. If, as the Examiners have asserted, Fire provided sufficient disclosure in the '559 patent to permit the dsRNA silencing of a target gene in all cells – both invertebrate and vertebrate - then why did the art subsequently recognize Tuschl *et al.* for work in 2001 as being the *first* use of dsRNA in a mammalian cell line? In fact, prior to my work in human cells, and 5 months later as published by Tuschl – no one in the art was able to demonstrate the silencing effect of dsRNA on a target gene in a *human* cell. Accordingly, the answer to these questions is simple – Fire's experiments in embryonic invertebrate cells simply did not, and could not, anticipate the target gene silencing effect of dsRNA in a human cell – regardless of the fact that claim 1 of the '559 patent failed to expressly state the understood species limitation to invertebrate cells.

22. The '559 claims do not expressly define the Fire invention as effective in a human cell. There is a good reason for what would otherwise be such an apparent oversight – Fire never believed that his invention would work in mammalian cells because he expected the intracellular defenses present in the mammalian cells to block translation of the foreign dsRNA. While the Fire patent may, in fact, be valid as to methods of treating cells of nematodes or invertebrate animals – the patent is simply not enabling for mammalian cells – nor could the patent expressly

claim a method that is effective in mammalian cells. The inventor never possessed such an invention – so he could not claim it or even describe how to overcome the expected problems. Fire never intended for his invention to actually be practiced in a vertebrate because he had no reason to believe that dsRNA could be introduced into viable mammalian cells. If the recognized problems were not already know at the time of the Fire invention, one might interpret the claims differently. But, to find that the Fire patent anticipated my invention would have required some teaching in either the '559 patent or in the prior art in 1997 or 1998, that would address the problem of intracellular defenses in a mammalian cell that were expected to attack the foreign dsRNA before it could act to silence the target gene. No such teaching was known or published AT THE FILING DATE OF THE FIRE '559 PATENT. The use of dsRNA in a mammalian cell was not discovered until years later. Paddison *et al.* were correct on page 1443, second column, when they stated that:

> The ability to apply RNAi in mammals will undoubtedly spark a firestorm of effort to assess the consequences of suppressing the expression of genes in cultured mammalian cells.

23. When Paddison *et al.* made that statement in 2002, the Fire work (as claimed in the '559 patent) had been well published, yet even four years after the filing date of the Fire patent, no one had been able to apply RNAi in a mammalian cell, even though many were trying, and everyone wanted to succeed to place themselves at the forefront of the expected "firestorm" of success and recognition. The goal was clear, but actually silencing a mammalian gene with RNAi had proven far more elusive than the methods claimed by Fire in an invertebrate cell, or than the present Patent Examiners appear to have recognized.

24. The evidence is unmistakable when one reviews the history of RNAi; Fire's work was not the beginning of RNAi in mammals. While an important early piece of the puzzle, Fire's work could only teach what he had discovered in 1997 and 1998 – which was the use of dsRNA in an embryonic invertebrate cell that offered no intracellular defenses. Beyond that, since he could not solve the problem of intracellular defenses, Fire and his co-inventor could only speculate what might finally be discovered years later when investigators finally could apply RNAi to mammals.

25. Contrary to Fire *et al.*, the specification for my patent application offers no speculation. I have provided working examples showing that RNAi has actually been induced in CHP 100 neuro-epithelioma (melanoma) and HL-60 leukemia cells, which represent two different human cancer cell lines from different tissues and developmental origins. Because the instant application shows that RNAi can be induced in such widely divergent human cancer cell lines, one skilled in the art would understand that RNAi can now be induced in any human cell. Thus, the present specification (including the working examples) contains ample direction for how to practice the full breadth of my claimed methods.

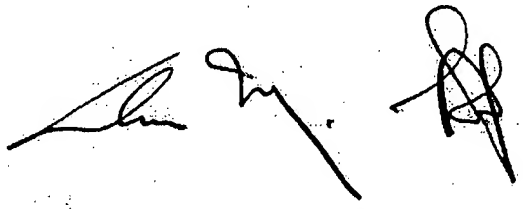
26. For example, the experiments described on page 12, line. 23 to page 14, line 6 of my present specification show that KitR expression was significantly reduced ($p<0.01$) in human cancer cells treated with 150 or 250 μg/ml c-Kit dsRNA. However, no reduction in KitR expression was seen upon treatment of the cells with control green fluorescent protein (GFP) dsRNA. If the inhibition of KitR expression upon administration of c-Kit dsRNA had been due to a general inhibition of translation via the PKR response, then a similar reduction in KitR expression would have been induced by the GFP dsRNA. However, since KitR expression was reduced only upon administration of the target specific c-Kit dsRNA, the inhibition of KitR expression was necessarily due to gene-specific gene silencing (disrupted expression) as a result of the introduced dsRNA. Thus, the present specification, in describing and enabling the claimed invention, demonstrates the feasibility of inducing gene-specific RNAi in human cells with dsRNA, without apparent interference from the PKR response. This has been argued at length in the recorded prosecution history.

27. Until my work prior to November 2000 demonstrated the use of dsRNA to silence or disrupt the expression of a target gene in a *human* cell – no one – and I repeat – no one had, to my knowledge, been able to apply RNAi in mature human cells. I have always believed that the purpose of the US Patent System is to recognize the inventor who first discovers a new and highly useful invention. I have done that, and I am seeking only the fair and just patent recognition for what I have accomplished.

28. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,



Date: September 14, 2005

Alan M. Gewirtz

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Patent application of Alan Gewirtz	
Serial No.: 09/993,183	Group Art Unit: 1635
Filed: November 14, 2001	Examiner: Jon B. Ashen
For: POST-TRANSCRIPTIONAL GENE SILENCING BY RNAi IN MAMMALIAN CELLS	Conf. No. 6995

Declaration of Dr. Alan M. Gewirtz for Earlier Date of Invention under 37 C.F.R. §1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Alan M. Gewirtz, declare that:

1. I am the sole inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 09/993,183, including all subject matter disclosed and claimed therein.

2. I am a Professor of Internal Medicine and Pathology-Laboratory Medicine at the University of Pennsylvania School of Medicine, Division of Hematology/Oncology. The Experiment set forth in the patent application, and all related experiments, were conducted in my laboratory and under my direction at the University of Pennsylvania in the United States of America.

3. Prior to August 3, 2000, Gokdan Demir, MD, working under my direction, began a series of experiments with me, on the project reported in US Patent Application 09/993,183, first filed as Provisional Patent Application 60/248,346, filed November 14, 2000. I initially summarized the concept of the invention on the page reproduced at Tab 1 attached hereto, with dates redacted. All dates are similarly redacted on the attached exhibits. No other change was

made to the document before copying and attaching the pages hereto. If the Examiner wishes to see the actual, dated documents, Applicant can comply, and has the entire notebook in which the pages were recorded in date-sequence order.

4. Initially, reliable protocols were optimized for isolating total RNA and for preparing double-stranded RNA (dsRNA). The human c-kit gene target has been well characterized in the literature, and previously used in other experiments in human cells in the Gewirtz laboratory. Throughout the course of these experiments, spectrophotometric analyses were conducted in the laboratory of Dr. Stephen G. Emerson at the University of Pennsylvania.

5. To begin, a portion of the 5' end of c-Kit genomic cDNA was subcloned into expression vector pcDNA3 (containing T7 and SP6 promoters) by digesting with BamH1. See Tab 2 attached hereto. Using the c-Kit gene as a target, the effect of dsRNA on the expression of the c-kit receptor (KitR) was evaluated in CHP 100 neuroepithelioma (human melanoma) cells and HL-60 (human leukemia) cells, both of which were known to express KitR. See Tabs 3 and 4 attached hereto. Cell lines were maintained in RPMI 1640 media (GibcoBRL, Gaithersburg, Md.) containing 10% BCS. Varying amounts (150-350 µg/ml) of Kit dsRNA (KdsRNA) were added to the culture media. See Tabs 3, 4 and 6-10 attached hereto.

6. As a control, GFP dsRNA (GdsRNA) was added. See Tabs 5 and 6 attached hereto. As a control, 724 bp of Green Fluorescent Protein (GFP) cDNA was subcloned into pcDNA3 by digesting with EcoRI and HindIII.

7. Subcloned vectors were amplified in a chemically competent strain of E. coli DH5_ cells. *In vitro* transcription reactions were carried out using known methods to linearize the plasmids using EcoRV and HindIII to synthesize the sense and corresponding antisense RNA strands, respectively. Digested plasmids were treated with Proteinase K to inactive any RNases, and purified as a template for transcription by commercial methods. RNA polymerases were from Promega. The products were pooled and annealed for 10 minutes at 90°C, 10 minutes at 4°C, and 2 hours at 40°C in a hybridization mixture containing NaCl 250 mM, Tris HCl 40 mM at pH 7.5 and EDTA 5 mM in RNase free water. The dsRNA was eluted using diethyl pyrocarbonate treated H_2O, and the integrity of the dsRNA was confirmed by running a 1% agarose gel in TBE 1X (90 mM Tris-borate/2 mM EDTA pH 8.0). It was then purified by column chromatography. See Tabs 6-7 attached hereto. See also, for example, the pages at Tab

8 showing an agarose gel showing the affect of dsRNA on c-Kit receptor signaling in HL-60 cells.

8. Cells were incubated under the same conditions, at 37°C., in 5% CO_2 for 1-4 days. See Tabs 8-10 attached hereto. At the end of the incubation period, FACS analyses were performed as described in the specification. Although no effect on KitR expression was observed until day 3, then inhibition was seen as disclosed in the specification, although only in the cells exposed to the KdsRNA. HL-60 cells behaved differently as shown at Tab 9, and as disclosed in the specification from CHP 100 cells, in both cases the KitR expression was unaffected by comparable doses of GdsRNA.

9. The foregoing experiment (actually a series of experiments) demonstrated for the first time that it is possible to disrupt gene expression at the mRNA level in a mammalian target gene in a *human* cell by providing to that human cell, interfering dsRNA (dsRNA) guide sequences that are homologous to a portion of the target gene, such that RNA interference (RNAi) of the target gene is induced. For example as shown, KitR expression was significantly reduced in human cancer cells treated with c-Kit dsRNA, but no reduction in KitR expression was seen upon treatment of the cells with GdsRNA. If the inhibition of KitR expression upon administration of c-Kit dsRNA had been due to a general inhibition of translation via the PKR response, then a similar reduction in KitR expression would have been induced by the GFP dsRNA. However, since KitR expression was reduced only upon administration of the target specific c-Kit dsRNA, the inhibition of KitR expression was necessarily due to gene-specific gene silencing (disrupted expression) as a result of the introduced dsRNA. Thus, the present specification, in describing and enabling the claimed invention, demonstrates the feasibility of inducing gene-specific RNAi in human cells with dsRNA, without apparent interference from the PKR response or other defenses.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,

Date: April 28, 2006

Alan M. Gewirtz

EXHIBIT 11

PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 7 : C12N 15/11, A61K 31/713

A1

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199 56 568.6	24. November 1999 (24.11.99)	DE

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(81) Bestimmungsstaaten: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht

Mit internationalem Recherchenbericht.

Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist; Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) Title: METHOD AND MEDICAMENT FOR INHIBITING THE EXPRESSION OF A DEFINED GENE

(54) Bezeichnung: VERFAHREN UND MEDIKAMENT ZUR HEMMUNG DER EXPRESSION EINES VORGEGEBENEN GENS

(57) Abstract

The invention relates to a medicament containing at least one double–stranded oligoribonucleotide (dsRNA) designed to inhibit the expression of a target gene. According to the invention, one strand of the dsRNA is at least in part complementary to the target gene.

(57) Zusammenfassung

Die Erfindung betrifft ein Medikament mit mindestens einem Oligoribonukleotid mit doppelsträniger Struktur (dsRNA) zur Hemmung der Expression eines Zielgens, wobei ein Strang der dsRNA zumindest abschnittsweise komplementär zum Zielgen ist.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

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DK	Dänemark	LK	Sri Lanka	SE	Schweden		
EE	Estland	LR	Liberia	SG	Singapur		

Verfahren und Medikament zur Hemmung der Expression eines vorgegebenen Gens

Die Erfindung betrifft Verfahren nach den Oberbegriffen der Ansprüche 1 und 2. Sie betrifft ferner ein Medikament und eine Verwendung doppelsträngiger Oligoribonukleotide sowie einen dafür kodierenden Vektor.

Ein solches Verfahren ist aus der nachveröffentlichten WO 99/32619 bekannt. Das bekannte Verfahren zielt auf die Hemmung der Expression von Genen in Zellen von Invertebraten ab. Dazu ist es erforderlich, daß das doppelsträngige Oligoribonukleotid eine zum Zielgen identische Sequenz mit einer Länge von mindestens 50 Basen aufweist. Zur Erzielung einer effizienten Hemmung ist eine Länge der identischen Sequenz von 300 bis 1000 Basenpaare erforderlich. Der Herstellungsaufwand eines solchen Oligoribonukleotids ist hoch.

Die DE 196 31 919 C2 beschreibt eine Anti-Sinn-RNA mit besonderen Sekundärstrukturen, wobei die Anti-Sinn-RNA in Form eines sie kodierenden Vektors vorliegt. Bei der Anti-Sinn-RNA handelt es sich um ein RNA-Molekül, das komplementär zu Bereichen der mRNA ist. Durch Bindung an diese Bereiche wird eine Hemmung der Genexpression bewirkt. Diese Hemmung kann insbesondere zur Diagnose und/oder Therapie von Erkrankungen, z.B. Tumorerkrankungen oder viralen Infektionen, eingesetzt werden. - Die Anti-Sinn-RNA muß nachteiligerweise in einer Menge in die Zelle eingebracht werden, die mindestens genauso groß wie die Menge der mRNA ist. Die Wirksamkeit der bekannten Anti-Sinn-Verfahren ist nicht besonders hoch.

Aus der US 5,712,257 ist ein Medikament bekannt, das fehlgepaarte doppelsträngige RNA (dsRNA) und biologisch aktive fehlgepaarte Bruchstücke von dsRNA in Form eines ternären Komple-

xes mit einem oberflächenaktiven Mittel enthält. Die dabei verwendete dsRNA besteht aus synthetisch hergestellten Nukleinsäureeinzelsträngen ohne definierte Basensequenz. Die Einzelstränge gehen nicht-reguläre, sogenannte "Nicht-Watson-Crick"-Basenpaarungen miteinander ein, so daß fehlgepaarte Doppelstränge gebildet werden. Die bekannte dsRNA dient zur Hemmung der Vermehrung von Retroviren, wie HIV. Die Vermehrung des Virus kann gehemmt werden, wenn nicht-sequenzspezifische dsRNA in die Zellen eingebracht wird. Es kommt dabei zu einer Induktion von Interferon, wodurch die Virusvermehrung gehemmt werden soll. Der hemmende Effekt bzw. die Wirksamkeit dieses Verfahrens ist gering.

Aus Fire, A. et.al, NATURE, Vol. 391, pp. 806 ist es bekannt, daß dsRNA, deren einer Strang abschnittsweise komplementär zu einem zu hemmenden Gen eines Fadenwurms ist, die Expression dieses Gens mit einer hohen Wirksamkeit hemmt. Es wird die Auffassung vertreten, daß die besondere Wirksamkeit der verwendeten dsRNA in Zellen des Fadenwurms nicht auf dem Anti-Sinn-Prinzip beruht, sondern möglicherweise auf katalytische Eigenschaften der dsRNA bzw. durch sie induzierte Enzyme zurückzuführen ist. - Über die Wirksamkeit spezifischer dsRNA in bezug auf die Hemmung der Genexpression, insbesondere in Säugerzellen und humanen Zellen, ist in diesem Artikel nichts ausgesagt.

Aufgabe der vorliegenden Erfindung ist es, die Nachteile nach dem Stand der Technik zu beseitigen. Es soll insbesondere ein möglichst effizientes Verfahren, Medikament bzw. eine möglichst effiziente Verwendung zur Herstellung eines Medikaments angegeben werden, mit dem/der eine besonders wirksame Hemmung der Expression eines vorgegebenen Zielgens bewirkbar ist.

Diese Aufgabe wird durch die Merkmale der Ansprüche 1, 2, 37, 38 und 74 und 75 gelöst. Vorteilhafte Ausgestaltungen ergeben sich aus den Ansprüchen 3 bis 36, 39 bis 73 und 76 bis 112.

Nach Maßgabe der verfahrensseitigen Erfindungen ist jeweils vorgesehen, daß der zum Zielgen komplementäre Bereich I höchstens 49 aufeinanderfolgenden Nukleotidpaare aufweist.

Erfindungsgemäß sind ein Oligoribonukleotid oder ein dafür kodierender Vektor vorgesehen. Das Oligoribonukleotid weist zumindest abschnittsweise eine definierte Nukleotidsequenz auf. Der definierte Abschnitt kann auf den komplementären Bereich I beschränkt sein. Es kann aber auch sein, daß das doppelsträngige Oligoribonukleotid insgesamt eine definierte Nukleotidsequenz aufweist.

Es hat sich überraschenderweise gezeigt, daß bereits bei einer Länge des komplementären Breichs I von höchstens 49 Basenpaaren eine wirksame Hemmung der Expression des Zielgens erreicht werden kann. Entsprechende Oligoribonukleotide können mit geringerem Herstellungsaufwand bereitgestellt werden.

Insbesondere dsRNA mit einer Länge von mehr als 50 Nukleotidpaaren induziert in Säugerzellen und humanen Zellen bestimmte zelluläre Mechanismen, z.B. die dsRNA-abhängige Proteinkinase oder das 2-5A-System. Das führt zum Verschwinden des durch die eine definierte Sequenz aufweisende dsRNA vermittelten Interferenzeffektes. Dadurch wird die Proteinbiosynthese in der Zelle blockiert. Insbesondere dieser Nachteil wird durch die vorliegende Erfindung beseitigt.

Weiterhin ist die Aufnahme von dsRNA mit kurzer Kettenlänge in die Zelle bzw. in den Zellkern gegenüber längerkettigen dsRNAs deutlich erleichtert.

Es hat sich als vorteilhaft erwiesen, daß die dsRNA oder der Vektor verpackt in micellare Strukturen, vorzugsweise in Liposomen, vorliegt. Die dsRNA oder der Vektor kann gleichfalls in virale natürliche Kapside oder in auf chemischem oder enzymatischem Weg hergestellte künstliche Kapside oder davon abgeleitete Strukturen eingeschlossen sein. - Die vorgenannten Merkmale ermöglichen ein Einschleusen der dsRNA bzw. des Vektors in vorgegebene Zielzellen.

Nach einem weiteren Ausgestaltungsmerkmal weist die dsRNA 10 bis 1000, vorzugsweise 15 bis 49, Basenpaare auf. Die dsRNA kann also länger als der zum Zielgen komplementäre Bereich I sein. Der komplementäre Bereich I kann endständig angeordnet oder in die dsRNA eingeschaltet sein. Eine solche dsRNA bzw. ein zur Kodierung derselben vorgesehener Vektor können synthetisch bzw. enzymatisch mit gängigen Verfahren hergestellt werden.

Das zu hemmende Gen wird zweckmäßigerweise in eukaryontischen Zellen exprimiert. Das Zielgen kann aus der folgenden Gruppe ausgewählt sein: Onkogen, Cytokin-Gen, Id-Protein-Gen, Entwicklungsgen, Priongen. Es kann auch in pathogenen Organismen, vorzugsweise in Plasmodien, exprimiert werden. Es kann Bestandteil eines, vorzugsweise humanpathogenen, Virus oder Viroids sein. - Das vorgeschlagene Verfahrenmöglicht die Herstellung von Mitteln zur Therapie genetisch gesteuerter Krankheiten, z.B. Krebs, viraler Erkrankungen oder Morbus Alzheimer.

Das Virus oder Viroid kann auch ein tier- oder planzenpathogenes Virus oder Viroid sein. In diesem Fall erlaubt das erfindungsgemäße Verfahren auch die Bereitstellung von Mitteln zur Behandlung von Tier- oder Pflanzenkrankheiten.

Nach einem weiteren Ausgestaltungsmerkmal ist die dsRNA abschnittsweise doppelsträngig ausgebildet. Ein innerhalb der doppelsträngigen Struktur komplementärer Bereich II wird aus zwei separaten RNA-Einzelsträngen oder aus selbstkomplementären Bereichen eines, vorzugsweise zirkulär ausgebildeten, topologisch geschlossenen RNA-Einzelstrangs gebildet.

Die Enden der dsRNA können modifiziert werden, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken. Eine Dissoziation tritt insbesondere bei Verwendung niedriger Konzentrationen oder kurzer Kettenlängen auf. Zur besonders wirksamen Hemmung der Dissoziation kann der durch die Nukleotidpaare bewirkte Zusammenhalt des komplementären Bereichs II durch mindestens eine, vorzugsweise zwei, weitere chemische Verknüpfung/en erhöht werden. - Eine erfindungsgemäße dsRNA, deren Dissoziation vermindert ist, weist eine höhere Stabilität gegen enzymatischen und chemischen Abbau in der Zelle bzw. im Organismus auf.

Insbesondere bei Verwendung eines erfindungsgemäßen Vektors kann der komplementäre Bereich II aus selbstkomplementären Bereichen einer RNA-Haarnadelschleife gebildet wird. Die Nukleotide sind im Schleifenbereich zwischen der doppelsträngigen Struktur zum Schutz vor Abbau zweckmäßigerweise chemisch modifiziert.

Die chemische Verknüpfung wird zweckmäßigerweise durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet. Sie kann nach einem besonders vorteilhaften Ausgestaltungsmerkmal an mindestens einem, vorzugs-

weise an beiden, Ende/n des komplementären Bereichs II hergestellt werden.

Es hat sich weiter als vorteilhaft erwiesen, daß die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Polyethylenglycol-Ketten sind. Die chemische Verknüpfung kann auch durch in den komplementären Bereichen II anstelle von Purinen benutzte Purinanaloga gebildet werden. Von Vorteil ist es ferner, daß die chemische Verknüpfung durch in den komplementären Bereichen II eingeführte Azabenzoleinheiten gebildet wird. Sie kann außerdem durch in den komplementären Bereichen II anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet werden.

Es hat sich als zweckmäßig erwiesen, daß zur Herstellung der chemischen Verknüpfung mindestens eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N`-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen. Ferner kann die chemische Verknüpfung durch an den Enden des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet werden. Vorzugsweise wird die chemische Verknüpfung an den Enden des doppelsträngigen Bereichs durch Tripelhelix-Bindungen hergestellt.

Die chemische Verknüpfung kann zweckmäßigerweise durch ultraviolettes Licht induziert werden.

Die Nukleotide der dsRNA können modifiziert sein. Dies wirkt einer Aktivierung einer von doppelsträngiger RNA abhängigen Proteinkinase, PKR, in der Zelle entgegen. Vorteilhafterweise ist mindestens eine 2´-Hydroxylgruppe der Nukleotide der dsRNA

in dem komplementären Bereich II durch eine chemische Gruppe, vorzugsweise eine 2′-Amino- oder eine 2′-Methylgruppe, ersetzt. Mindestens ein Nukleotid in mindestens einem Strang des komplementären Bereichs II kann auch ein sogenanntes "locked nucleotide" mit einem, vorzugsweise durch eine 2'-O, 4'-C-Methylenbrücke, chemisch modifizierten Zuckerring sein. Vorteilhafterweise sind mehrere Nukleotide "locked nucleotides".

Nach einer weiteren besonders vorteilhaften Ausgestaltung ist vorgesehen, daß die dsRNA oder der Vektor an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben wird. Das Hüllprotein kann vom Polyomavirus abgeleitet sein. Es kann das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthalten. Die Verwendung derartiger Hüllproteine ist z.B. aus der DE 196 18 797 A1 bekannt, deren Offenbarungsgehalt hiermit einbezogen wird. - Die vorgenannten Merkmale erleichtert wesentlich das Einführen der dsRNA bzw. des Vektors in die Zelle.

Vorzugsweise ist bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt. Das gebildete Konstrukt ist besonders stabil.

Die dsRNA kann zum primären oder prozessierten RNA-Transkript des Zielgens komplementär sein.- Die Zelle kann eine Vertebratenzelle oder eine menschliche Zelle sein.

Es können mindestens zwei voneinander verschiedene dsRNAs oder mindestens ein dafür kodierender Vektor in die Zelle eingeführt werden, wobei ein Strang jeder dsRNA zumindest abschnittsweise komplementär zu jeweils einem von mindestens

zwei verschiedenen Zielgenen ist. Dadurch ist es möglich gleichzeitig die Expression mindestens zwei verschiedener Zielgene zu hemmen. Um die Expression einer von doppelsträngiger RNA abhängigen Proteinkinase, PKR, in der Zelle zu unterdrücken, ist eines der Zielgene vorteilhafterweise das PKR-Gen. Dadurch kann die PKR-Aktivität in der Zelle wirksam unterdrückt werden.

Nach Maßgabe der Erfindung ist ferner ein Medikament mit mindestens einem Oligoribonukleotid mit doppelsträngiger Struktur (dsRNA) zur Hemmung der Expression eines vorgegebenen Zielgens vorgesehen, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist. - Es hat sich überraschend gezeigt, daß eine solche dsRNA sich als Medikament zur Hemmung der Expression eines vorgegebenen Gens in Säugerzellen eignet. Die Hemmung wird im Vergleich zur Verwendung einzelsträngiger Oligoribonukleotide bereits bei Konzentrationen bewirkt, die um mindestens eine Größenordnung niedriger sind. Das erfindungsgemäße Medikament ist hoch wirksam. Es sind geringere Nebenwirkungen zu erwarten.

Nach weiterer Maßgabe der Erfindung ist ein Medikament mit mindestens einem Vektor zur Kodierung mindestens eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Hemmung der Expression eines vorgegebenen Zielgens vorgesehen, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist. - Das vorgeschlagene Medikament weist die vorgenannten Vorteile auf. Durch die Verwendung eines Vektors können insbesondere Herstellungskosten eingespart werden.

Nach einer besonders vorteilhaften Ausgestaltung weist der komplementäre Bereich I höchstens 49 aufeinanderfolgende Nukleotidpaare auf. - Es hat sich überraschenderweise gezeigt,

daß bereits bei einer Länge des komplementären Bereichs I von höchstens 49 Basenpaaren eine effiziente Hemmung der Expression des Zielgens erreicht werden kann. Entsprechende Oligoribonukleotide können mit geringerem Herstellungsaufwand bereitgestellt werden.

Nach weiterer Maßgabe der Erfindung ist eine Verwendung eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Herstellung eines Medikaments zur Hemmung der Expression eines vorgegebenen Zielgens vorgesehen, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist. - Überraschenderweise eignet sich eine solche dsRNA zur Herstellung eines Medikaments zur Hemmung der Expression eines vorgegebenen Gens. Bei einer Verwendung von dsRNA wird die Hemmung im Vergleich zur Verwendung einzelsträngiger Oligoribonukleotide schon bei um eine Größenordnung geringeren Konzentrationen bewirkt. Die erfindungsgemäße Verwendung ermöglicht also die Herstellung besonders wirksamer Medikamente.

Nach weiterer Maßgabe der Erfindung ist die Verwendung eines Vektors zur Kodierung mindestens eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Herstellung eines Medikaments zur Hemmung der Expression eines vorgegebenen Zielgens vorgesehen, wobei ein Strang der dsRNA einen zu diesem Zielgen zumindest abschnittsweise komplementären Bereich I aufweist. - Die Verwendung eines Vektors ermöglicht eine besonders wirksame Gentherapie.

Hinsichtlich vorteilhafter Ausgestaltungen des Medikaments und der Verwendung wird auf die Beschreibung der vorangegangenen Merkmale verwiesen.

Nachfolgend werden anhand der Figuren Ausführungsbeispiele der Erfindung näher erläutert. Es zeigen:

Fig. 1 die schematische Darstellung eines Plasmids für die *in vitro*-Transkription mit T7- und SP6-Polymerase,

Fig. 2 RNA nach Elektrophorese auf einem 8%igen Polyacrylamidgel und Ethidiumbromidfärbung,

Fig. 3 eine Darstellung radioaktiver RNA-Transkripte nach Elektrophorese auf einem 8%igen Polyacrylamidgel mit 7 M Harnstoff mittels eines "Instant Imagers" und

Fig. 4 a - e Texas-Rot- und YFP-Fluoreszenz in murinen Fibroblasten.

Ausführungsbeispiel 1:

Die Inhibition der Transkription wurde durch sequenzhomologe dsRNA in einem *in vitro*-Transkriptionssystem mit einem Kernextrakt aus humanen HeLa-Zellen nachgewiesen. Die DNA-Matrize für diesen Versuch war das mittels *Bam*HI linearisierte Plasmid pCMV1200.

Herstellung der Matrizenplasmide:

Zur Verwendung bei der enzymatischen Synthese der dsRNA wurde das in Fig. 1 dargestellte Plasmid konstruiert. Dazu wurde zunächst eine Polymerase-Kettenreaktion (PCR) mit der "positive control DNA" des HeLaScribe® Nuclear Extract *in vitro* Transkriptionskits der Firma Promega, Madison, USA als DNA-Matrize durchgeführt. Einer der verwendeten Primer enthielt die Sequenz einer *Eco*RI-Schnittstelle und des T7-RNA-Polymerase-Promotors gemäß Sequenzprotokoll Nr. 1. Der andere Primer ent-

hielt die Sequenz einer *Bam*HI-Schnittstelle und des SP6-RNA-Polymerase-Promotors gemäß Sequenzprotokoll Nr. 2. Darüber hinaus wiesen beide Primer an ihren 3'-Enden identische bzw. komplementäre Bereiche zur DNA-Matrize auf. Die PCR wurde mittels des "*Taq PCR Core Kits*" der Firma Qiagen, Hilden, Deutschland nach Herstellerangaben durchgeführt. In einem Volumen von 100 µl wurden 1,5 mM $MgCl_2$, je 200 µM dNTP, je 0,5 µM Primer, 2,5 U *Taq*-DNA-Polymerase und etwa 100 ng "positive control DNA" als Matrize in PCR-Puffer eingesetzt. Nach der anfänglichen Denaturierung der Matrizen-DNA durch Erhitzen auf 94°C für 5 Minuten erfolgte die Amplifikation in 30 Zyklen von je 60 Sekunden Denaturierung bei 94°C, 60 Sekunden Annealing bei 5°C unterhalb der berechneten Schmelztemperatur der Primer und 1,5 - 2 Minuten Polymerisation bei 72°C. Nach einer Schlußpolymerisation von 5 Minuten bei 72°C wurden 5 µl des Reaktionsansatzes durch Agarosegelelektrophorese analysiert. Die Länge des so amplifizierten DNA-Fragmentes betrug 400 Basenpaare, wobei 340 Basenpaare der "positive control DNA" entsprachen. Das PCR-Produkt wurde aufgereinigt, mit *Eco*RI und *Bam*HI hydrolysiert und nach erneuter Aufreinigung zur Ligation mit einem ebenfalls durch *Eco*RI und *Bam*HI hydrolysierten pUC18 Vektor eingesetzt. Es erfolgte Transformation von *E. coli* XL1-blue. Das erhaltene Plasmid (pCMV5) trägt ein DNA-Fragment, das am 5´-Ende von dem T7- und am 3´-Ende von dem SP6-Promotor flankiert wird. Durch Linearisierung des Plasmids mit *Bam*HI kann es *in vitro* mit der T7-RNA-Polymerase zur *run-off*-Transkription einer 340 Nukleotide langen, in Sequenzprotokoll Nr. 3 dargestellten, einzelsträngigen RNA eingesetzt werden. Wird das Plasmid mit *Eco*RI linearisiert, kann es zur *run-off*-Transkription mit der SP6-RNA-Polymerase eingesetzt werden, wobei der komplementäre Strang entsteht. Entsprechend dem zuvor dargestellten Verfahren wurde auch eine 23 Nukleotide längere RNA synthetisiert. Dazu wurde eine in Sequenzprotokoll

Nr. 4 dargestellte DNA über die *Eco*RI und *Bam*HI-Schnittstellen mit dem pUC18 Vektor ligiert.

Als DNA-Matrize für die in vitro-Transkription mit HeLa-Kernextrakt wurde das Plasmid pCMV1200 konstruiert. Dazu wurde ein 1191 bp großes EcoRI/BamHI-Fragment der im HeLaScribe® Nuclear Extract *in vitro* Transkriptionskit enthaltenen Positivkontroll-DNA mittels PCR amplifiziert. Das amplifizierte Fragment umfaßt den 828 bp großen "unmittelbar frühen" CMV-Promotor und ein 363 bp großes transkribierbares DNA-Fragment. Das PCR-Produkt wurde über "T-Überhang"-Ligation mit dem Vektor pGEM-T ligiert. Am 5'-Ende des Fragments ist eine BamHI-Schnittstelle. Das Plasmid wurde durch Hydrolyse mit BamHI linearisiert und als Matrize zur run-off-Transkription eingesetzt.

in vitro-Transkription der komplementären Einzelstränge:

pCMV5-Plasmid-DNA wurde mit *Eco*RI bzw. *Bam*HI linearisiert. Sie wurde als DNA-Matrize für eine *in vitro*-Transkription der komplementären RNA-Einzelstränge mit SP6- bzw. T7-RNA-Polymerase verwendet. Dazu wurde das "Riboprobe *in vitro* Transcription" System der Firma Promega, Madison, USA eingesetzt. Nach Herstellerangaben wurden 2 µg linearisierte Plasmid-DNA in 100 µl Transkriptionspuffer und 40 U T7- oder SP6-RNA-Polymerase 5 - 6 Stunden bei 37°C inkubiert. Anschließend wurde die DNA-Matrize durch Zugabe von 2,5 µl RNase-freier DNase RQ1 und Inkubation für 30 Minuten bei 37°C abgebaut. Der Transkriptionsansatz wurde mit H_2O auf 300 µl aufgefüllt und durch Phenolextraktion gereinigt. Die RNA wurde durch Zugabe von 150 µl 7 M Ammoniumacatat und 1125 µl Ethanol gefällt und bis zur Hybridisierung bei -65°C aufbewahrt.

Herstellung der RNA-Doppelstränge:

Zur Hybridisierung wurden 500 µl der in Ethanol aufbewahrten und gefällten einzelsträngigen RNA abzentrifugiert. Das resultierende Pellet wurde getrocknet und in 30 µl PIPES-Puffer, pH 6,4 in Gegenwart von 80 % Formamid, 400 mM NaCl und 1 mM EDTA aufgenommen. Jeweils 15 µl der komplementären Einzelstränge wurden zusammengegeben und für 10 Minuten auf 85°C erhitzt. Anschließend wurden die Ansätze bei 50°C über Nacht inkubiert und auf Raumtemperatur abgekühlt.

Bei der Hybridisierung wurden nur annähernd äquimolare Mengen der beiden Einzelstränge eingesetzt. Dadurch enthielten die dsRNA-Präparationen einzelsträngige RNA (ssRNA) als Kontamination. Um diese ssRNA-Kontaminationen zu entfernen, wurden die Ansätze nach der Hybridisierung mit den einzelstrangspezifischen Ribonukleasen RNase A aus Rinderpankreas und RNase T1 aus *Aspergillus oryzae* behandelt. RNase A ist eine für Pyrimidine spezifische Endoribonuklease. RNase T1 ist eine Endoribonuklease, die bevorzugt auf der 3´-Seite von Guanosinen schneidet. dsRNA ist kein Substrat für diese Ribonukleasen. Für die RNase-Behandlung wurde zu den Ansätzen in 300 µl Tris, pH 7,4, 300 mM NaCl und 5 mM EDTA 1,2 µl RNaseA in einer Konzentration von 10 mg/ml und 2 µl RNaseT1 in einer Konzentration von 290 µg/ml zugegeben. Die Ansätze wurden 1,5 Stunden bei 30°C inkubiert. Danach wurden die RNasen durch Zugabe von 5 µl Proteinase K in einer Konzentration von 20 mg/ml sowie 10 µl 20%iges SDS und Inkubation für 30 Minuten bei 37°C denaturiert. Die dsRNA wurde durch Phenol-Extraktion gereinigt und mit Ethanol gefällt. Um die Vollständigkeit des RNase-Verdaus überprüfen zu können, wurden zwei Kontrollansätze mit ssRNA analog zu den Hybridisierungsansätzen behandelt.

Das getrocknete Pellet wurde in 15 µl TE-Puffer, pH 6,5 aufgenommen und auf einem 8%igen Gel einer nativen Polyacrylamidgelelektrophorese unterzogen. Das Acrylamidgel wurde anschlie-

ßend in einer Ethidiumbromidlösung gefärbt und in einem Wasserbad gespült. Fig. 2 zeigt die auf einem UV-Transilluminator sichtbar gemachte RNA. Die auf Spur 1 aufgetragene *sense*- und die auf Spur 2 aufgetragene *antisense*-RNA zeigten unter den gewählten Bedingungen ein anderes Laufverhalten als die auf Spur 3 aufgetragene dsRNA des Hybridisierungsansatzes. Die auf den Spuren 4 bzw. 5 aufgetragene RNase-behandelte *sense*- bzw *antisense*-RNA erzeugte keine sichtbare Bande. Dies zeigt, daß die einzelsträngigen RNAs vollständig abgebaut wurden. Die auf Spur 6 aufgetragene RNase-behandelte dsRNA des Hybridisierungsansatzes ist resistent gegenüber der RNase-Behandlung. Die im nativen Gel im Vergleich zu der auf Spur 3 aufgetragenen dsRNA schneller wandernde Bande resultiert aus dsRNA, die frei von ssRNA ist. Neben der dominierenden Hauptbande treten nach der RNase-Behandlung schwächere, schneller wandernde Banden auf.

in vitro-Transkriptions-Test mit menschlichem Zellkernextrakt:

Unter Verwendung des HeLaScribe® Nuclear Extract *in vitro* Transkriptionskits der Firma Promega, Madison, USA wurde die Transkriptionseffizienz des oben angegebenen, im Plasmid pCMV1200 enthaltenen, zur "positive control DNA" homologen DNA-Fragments in Gegenwart der sequenzhomologen dsRNA (dsRNA-CMV5) bestimmt. Außerdem wurde der Einfluß der nicht-sequenzhomologen, dem "Gelb fluoreszierenden Protein" (YFP)-Gen entsprechenden dsRNA (dsRNA-YFP) untersucht. Diese dsRNA war analog zur sequenzhomologen dsRNA hergestellt worden. Die Sequenz eines Stranges dieser dsRNA ist Sequenzprotokoll Nr. 5 zu entnehmen. Als Matrize für die *run-off*-Transkription diente das Plasmid pCMV1200. Es trägt den "unmittelbar frühen" Promotor des Cytomegalievirus, der von der eukaryotischen RNA-Polymerase II erkannt wird, und ein transkribierbares DNA-Fragment. Die Transkription erfolgte mittels des HeLa-Kernextrakts, der alle notwendigen Proteine für eine Tran-

skription enthält. Durch Zugabe von [•-^{32}P]rGTP zum Transkriptionsansatz wurde radioaktiv markiertes Transkript erhalten. Das verwendete [•-^{32}P]rGTP hatte eine spezifische Aktivität von 400 Ci/mmol, 10 mCi/ml. Pro Ansatz wurden 3 mM $MgCl_2$, je 400 µM rATP, rCTP, rUTP, 16 µM rGTP, 0,4 µM [•-^{32}P]rGTP und je nach Versuch 1 fmol linearisierte Plasmid-DNA und verschiedene Mengen an dsRNA in Transkriptionspuffer eingesetzt. Jeder Ansatz wurde mit H_2O auf ein Volumen von 8,5 µl aufgefüllt. Die Ansätze wurden vorsichtig gemischt. Zum Starten der Transkription wurden 4 U HeLa-Kernextrakt in einem Volumen von 4 µl zugegeben und für 60 Minuten bei 30°C inkubiert. Die Reaktion wurde durch Zugabe von 87,5 µl auf 30°C erwärmten Stopp-Mix beendet. Zur Entfernung der Proteine wurden die Ansätze mit 100 µl Phenol/Chloroform/Isoamylalkohol (25:24:1, v/v/v), gesättigt mit TE-Puffer, pH 5,0, versetzt und 1 Minute kräftig gemischt. Zur Phasentrennung wurde etwa 1 Minute bei 12000 rpm zentrifugiert und die obere Phase in ein neues Reaktionsgefäß überführt. Zu jedem Ansatz wurden 250 µl Ethanol zugegeben. Die Ansätze wurden gut gemischt und für mindestens 15 Minuten auf Trockeneis/Methanol inkubiert. Zur Präzipitation der RNA wurden die Ansätze 20 Minuten bei 12000 rpm und 4°C zentrifugiert. Der Überstand wurde verworfen. Das Pellet wurde 15 Minuten im Vakuum getrocknet und in 10 µl H_2O resuspendiert. Zu jedem Ansatz wurden 10 µl denaturierender Probenpuffer zugegeben. Die Trennung des freien GTP vom entstandenen Transkript erfolgte mittels denaturierender Polyacrylamid-Gelelektrophorese auf einem 8%igen Gel mit 7 M Harnstoff. Die bei der Transkription mit HeLa-Kernextrakt gebildeten RNA-Transkripte in denaturierendem Probenpuffer wurden für 10 Minuten auf 90°C erhitzt und 10 µl davon sofort in die frisch gespülten Probentaschen aufgetragen. Die Elektrophorese erfolgte bei 40 mA. Die Menge der bei der Transkription gebildeten radioaktiven ssRNA wurde nach der Elektrophorese mit Hilfe eines *Instant Imager* analysiert.

Fig. 3 zeigt die mittels des *Instant Imagers* dargestellte radioaktive RNA aus einem repräsentativen Tests. Es wurden aus folgenden Transkriptionsansätzen gewonne Proben aufgetragen:

Spur 1: ohne Matrizen-DNA, ohne dsRNA;
Spur 2: 50 ng Matrizen-DNA, ohne dsRNA;
Spur 3: 50 ng Matrizen-DNA, 0,5 µg dsRNA-YFP;
Spur 4: 50 ng Matrizen-DNA, 1,5 µg dsRNA-YFP;
Spur 5: 50 ng Matrizen-DNA, 3 µg dsRNA-YFP;
Spur 6: 50 ng Matrizen-DNA, 5 µg dsRNA-YFP;
Spur 7: ohne Matrizen-DNA, 1,5 dsRNA-YFP;
Spur 8: 50 ng Matrizen-DNA, ohne dsRNA;
Spur 9: 50 ng Matrizen-DNA, 0,5 µg dsRNA-CMV5;
Spur 10: 50 ng Matrizen-DNA, 1,5 µg dsRNA-CMV5;
Spur 11: 50 ng Matrizen-DNA, 3 µg dsRNA-CMV5;
Spur 12: 50 ng Matrizen-DNA, 5 µg dsRNA-CMV5;

Es zeigte sich eine deutliche Verringerung der Menge an Transkript in Gegenwart von sequenzhomologer dsRNA im Vergleich zum Kontrollansatz ohne dsRNA sowie auch zu den Ansätzen mit nicht-sequenzhomologer dsRNA-YFP. Die Positivkontrolle in Spur 2 zeigt, daß bei der *in vitro*-Transkription mit HeLa-Kernextrakt radioaktives Transkript gebildet wurde. Der Ansatz dient zum Vergleich mit den Transkriptionsansätzen, die in Gegenwart von dsRNA inkubiert worden waren. Die Spuren 3 bis 6 zeigen, daß die Zugabe von nicht-sequenzspezifischer dsRNA-YFP keinen Einfluß auf die Menge des gebildeten Transkripts hat. Die Spuren 9 bis 12 zeigen, daß die Zugabe einer zwischen 1,5 und 3 µg liegenden Menge sequenzspezifischer dsRNA-CMV5 zu einer Abnahme der gebildeten Transkript-Menge führt. Um auszuschließen, daß die beobachteten Effekte nicht auf der dsRNA, sondern auf einer möglicherweise bei der Herstellung der dsRNA unabsichtlich mitgeführten Kontamination beruhen, wurde eine

weitere Kontrolle durchgeführt. Einzelstrang-RNA wurde wie oben beschrieben transkribiert und anschließend der RNase-Behandlung unterzogen. Mittels nativer Polyacrylamidgelelektrophorese konnte gezeigt werden, daß die ssRNA vollständig abgebaut worden war. Dieser Ansatz wurde wie die Hybridisierungsansätze einer Phenolextraktion und einer Ethanolfällung unterzogen und anschließend in TE-Puffer aufgenommen. Auf diese Weise wurde eine Probe erhalten, die keine RNA enthielt, aber mit den gleichen Enzymen und Puffern behandelt worden war wie die dsRNA. Spur 8 zeigt, daß der Zusatz dieser Probe keinen Einfluß auf die Transkription hatte. Die Abnahme des Transkripts bei Zugabe sequenzspezifischer dsRNA kann deshalb eindeutig der dsRNA selbst zugeschrieben werden. Die Reduzierung der Transkript-Menge eines Gens in Gegenwart von dsRNA bei einem menschlichen Transkriptionssystem zeigt eine Hemmung der Expression des entsprechenden Gens an. Dieser Effekt ist auf einen neuartigen, durch die dsRNA bedingten Mechanismus zurückzuführen.

Ausführungsbeispiel 2:

Als Testsystem für diese *in vivo*-Experimente diente die murine Fibroblasten-Zellinie NIH3T3, ATCC CRL-1658. Mit Hilfe der Mikroinjektion wurde das YFP-Gen in die Zellkerne eingebracht. Die Expression des YFP wurde unter dem Einfluß gleichzeitig mittransfizierter sequenzhomologer dsRNA untersucht. Diese dsRNA-YFP ist über eine Länge von 315 bp zum 5'-Bereich des YFP-Gens homolog. Die Nukleotidsequenz eines Strangs der dsRNA-YFP ist in Sequenzprotokoll Nr. 5 wiedergegeben. Die Auswertung unter dem Fluoreszenzmikroskop erfolgte 3 Stunden nach Injektion anhand der grün-gelben Fluoreszenz des gebildeten YFP.

Konstruktion des Matrizenplasmids und Herstellung der dsRNA:

Als Matrize für die Herstellung der YFP-dsRNA mittels T7- und SP6-*in vitro*-Transkription wurde ein Plasmid nach dem gleichen Prinzip wie im Ausführungsbeispiel 1 beschrieben konstruiert. Das gewünschte Genfragment wurde unter Verwendung des Primers *Eco*_T7_YFP gemäß Sequenzprotokoll Nr. 6 und *Bam*_SP6_YFP gemäß Sequenzprotokoll Nr. 7 mittels PCR amplifiziert und analog zu der obigen Beschreibung zur Herstellung der dsRNA verwendet. Die erhaltene dsRNA-YFP ist identisch mit der in Ausführungsbeispiel 1 als nicht-sequenzspezifische Kontrolle verwendeten dsRNA.

Es wurde eine am 3´-Ende der RNA gemäß Sequenzprotokoll Nr. 8 über eine C18-Linkergruppe chemisch mit dem 5´-Ende der komplementären RNA verknüpfte dsRNA (L-dsRNA) hergestellt. Dazu wurden mit Disulfid-Brücken modifizierte Synthone verwendet. Das 3´-terminale Synthon ist über den 3´-Kohlenstoff mit einer aliphatischen Linker-Gruppe über eine Disulfidbrücke an den festen Träger gebunden. Bei dem zum 3´-terminalen Synthon des einen Oligoribonukleotids komplementären 5´-terminalen Synthon des komplementären Oligoribonukleotids ist die 5´-Tritylschutzgruppe über einen weiteren aliphatischen Linker und eine Disulfidbrücke gebunden. Nach Synthese der beiden Einzelstränge, Entfernen der Schutzgruppen und Hybridisierung der komplementären Oligoribonukleotide gelangen die entstehenden Thiolgruppen in räumliche Nachbarschaft zueinander. Durch Oxidation werden die Einzelstränge über ihre aliphatischen Linker und eine Disulfidbrücke miteinander verknüpft. Anschließend erfolgt Reinigung mit Hilfe der HPLC.

Vorbereitung der Zellkulturen:

Die Zellen wurden in DMEM mit 4,5 g/l Glucose, 10 % fötalem Rinderserum unter 7,5 % CO_2-Atmosphäre bei 37°C in Kulturschalen inkubiert und vor Erreichen der Konfluenz passagiert. Das

Ablösen der Zellen erfolgte mit Trypsin/EDTA. Zur Vorbereitung der Mikroinjektion wurden die Zellen in Petrischalen überführt und bis zu Bildung von Mikrokolonien weiter inkubiert.

Mikroinjektion:

Die Kulturschalen wurde zur Mikroinjektion für ca. 10 Minuten aus dem Inkubator genommen. Es wurde in ca. 50 Zellkerne pro Ansatz innerhalb eines markierten Bereichs unter Verwendung des Mikroinjektionssystems AIS der Firma Carl Zeiss, Göttingen, Deutschland einzeln injiziert. Anschließend wurden die Zellen weitere drei Stunden inkubiert. Für die Mikroinjektion wurden Borosilikat-Glaskapillaren der Firma Hilgenberg GmbH, Malsfeld, Deutschland mit einem Spitzendurchmesser unter 0,5 µm vorbereitet. Die Mikroinjektion wurde mit einem Mikromanipulator der Firma Narishige Scientific Instrument Lab., Tokyo, Japan durchgeführt. Die Injektionsdauer betrug 0,8 Sekunden, der Druck ca. 100 hPa. Für die Transfektion wurde das Plasmid pCDNA-YFP verwendet, das ein ca. 800 bp großes *Bam*HI/*Eco*RI-Fragment mit dem Gen des YFP im Vektor pcDNA3 enthält. Die in die Zellkerne injizierten Proben enthielten 0,01 µg/µl pCDNA-YFP sowie an Dextran-70000 gekoppeltes Texas-Rot in 14 mM NaCl, 3 mM KCl, 10 mM KPO_4, pH 7,5. Zusätzlich wurden ca. 100 pl RNA mit einer Konzentration von 1 µM, bzw. 375 µM im Fall der L-dsRNA, zugegeben.

Die Zellen wurden bei Anregung mit Licht der Anregungswellenlänge von Texas-Rot, 568 nm, bzw. von YFP, 488 nm, mittels eines Fluoreszenzmikroskops untersucht. Einzelne Zellen wurden mittels einer digitalen Kamera dokumentiert. Die Figuren 4 a - e zeigen das Ergebnis für NIH3T3-Zellen. Bei den in Fig. 4 a gezeigten Zellen ist *sense*-YFP-ssRNA, in Fig. 4 b *antisense*-YFP-ssRNA, in Fig. 4 c dsRNA-YFP, in Fig. 4 d keine RNA und in Fig. 4 e L-dsRNA injiziert worden.

Das jeweils linke Feld zeigt die Fluoreszenz von Zellen, die mit 568 nm angeregt wurden. Rechts ist die Fluoreszenz derselben Zellen bei Anregung mit 488 nm zu sehen. Die Texas-Rot-Fluoreszenz aller dargestellten Zellen zeigt, daß die Injektionslösung erfolgreich in die Zellkerne appliziert wurde und getroffene Zellen nach drei Stunden noch lebendig waren. Abgestorbene Zellen zeigten keine Texas-Rot-Fluoreszenz mehr.

Die jeweils rechten Felder der Figuren 4 a und 4 b zeigen, daß die Expression des YFP bei Injektion der einzelsträngigen RNA in die Zellkerne nicht sichtbar inhibiert wurde. Das rechte Feld der Fig. 4 c zeigt Zellen, deren YFP-Fluoreszenz nach Injektion von dsRNA-YFP nicht mehr nachweisbar war. Fig. 4 d zeigt als Kontrolle Zellen, in die keine RNA injiziert worden war. Die in Fig. 4 e dargestellte Zelle zeigt durch die Injektion der L-dsRNA, die zum YFP-Gen sequenzhomologe Bereiche aufweist, eine nicht mehr nachweisbare YFP-Fluoreszenz. Dieses Ergebnis belegt, daß auch kürzere dsRNAs zur spezifischen Inhibition der Genexpression bei Säugern verwendet werden können, wenn die Doppelstränge durch chemische Verknüpfung der Einzelstränge stabilisiert werden.

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Patentansprüche

1. Verfahren zur Hemmung der Expression eines vorgegebenen Zielgens in einer Zelle, wobei ein Oligoribonukleotid mit doppelsträngiger Struktur (dsRNA) in die Zelle eingeführt wird, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist, **dadurch gekennzeichnet**, daß der zum Zielgen komplementäre Bereich I höchstens 49 aufeinanderfolgende Nukleotidpaare aufweist.

2. Verfahren zur Hemmung der Expression eines vorgegebenen Zielgens in einer Zelle, wobei ein Vektor zur Kodierung mindestens eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) in die Zelle eingeführt wird, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist, **dadurch gekennzeichnet**, daß der zum Zielgen komplementäre Bereich I höchstens 49 aufeinanderfolgende Nukleotidpaare aufweist.

3. Verfahren nach Anspruch 1 oder 2, wobei die dsRNA oder der Vektor in micellare Strukturen, vorzugsweise in Liposomen, eingeschlossen wird.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA oder der Vektor in virale natürliche Kapside oder in auf chemischem oder enzymatischem Weg hergestellte künstliche Kapside oder davon abgeleitete Strukturen eingeschlossen wird.

5. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA 10 bis 1000, vorzugsweise 15 bis 49, Basenpaare aufweist.

6. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen in eukaryontischen Zellen exprimiert wird.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen aus der folgenden Gruppe ausgewählt ist: Onkogen, Cytokin-Gen, Id-Protein-Gen, Entwicklungsgen, Priongen.

8. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimiert wird.

9. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

10. Verfahren nach Anspruch 9, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

11. Verfahren nach Anspruch 9, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

12. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA abschnittsweise doppelsträngig ausgebildet ist.

13. Verfahren nach einem der vorhergehenden Ansprüche, wobei ein innerhalb der doppelsträngigen Struktur komplementärer Bereich II aus zwei separaten RNA-Einzelsträngen oder aus selbstkomplementären Bereichen eines, vorzugsweise zirkulär ausgebildeten, topologisch geschlossenen RNA-Einzelstrangs gebildet wird.

14. Verfahren nach einem der vorhergehenden Ansprüche, wobei der komplementäre Bereich II aus selbstkomplementären Bereichen einer RNA-Haarnadelschleife gebildet wird.

15. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Nukleotide im Schleifenbereich zwischen der doppelsträngigen Struktur zum Schutz vor Abbau chemisch modifiziert sind.

16. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Enden der dsRNA modifiziert werden, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

17. Verfahren nach einem der vorhergehenden Ansprüche, wobei der durch die Nukleotidpaare bewirkte Zusammenhalt des komplementären Bereichs II durch mindestens eine, vorzugsweise zwei, weitere chemische Verknüpfung/en erhöht wird.

18. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet wird.

19. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung an mindestens einem, vorzugsweise an beiden, Enden des komplementären Bereichs II hergestellt wird.

20. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet wird, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Polyethylenglycol-Ketten sind.

21. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Purinen benutzten Purinanaloga gebildet wird.

22. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch in den komplementären Bereichen II eingeführte Azabenzoleinheiten gebildet wird.

23. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet wird.

24. Verfahren nach einem der vorhergehenden Ansprüche, wobei zur Herstellung der chemischen Verknüpfung mindestenes eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N`-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

25. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung durch an den Enden des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet wird.

26. Verfahren nach einem der vorhergehenden Ansprüche, wobei die chemische Verknüpfung an den Enden des doppelsträngigen Bereichs durch Tripelhelix-Bindungen hergestellt wird.

27. Verfahren nach einem der vorhergehenden Ansprüche, wobei mindestens eine 2´-Hydroxylgruppe der Nukleotide der

dsRNA in dem komplementären Bereich II durch eine chemische Gruppe, vorzugsweise eine 2´-Amino- oder eine 2´-Methylgruppe, ersetzt ist.

28. Verfahren nach einem der vorhergehenden Ansprüche, wobei mindestens ein Nukleotid in mindestens einem Strang des komplementären Bereichs II ein "locked nucleotide" mit einem, vorzugsweise durch eine 2'-O, 4'-C-Methylenbrücke, chemisch modifizierten Zuckerring ist.

29. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA oder der Vektor an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben wird.

30. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

31. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

32. Verfahren nach einem der vorhergehenden Ansprüche, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

33. Verfahren nach einem der vorhergehenden Ansprüche, wobei die dsRNA zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

34. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

35. Verfahren nach einem der vorhergehenden Ansprüche, wobei mindestens zwei voneinander verschiedene dsRNAs oder mindestens ein dafür kodierender Vektor in die Zelle eingeführt werden, wobei ein Strang jeder dsRNA zumindest abschnittsweise komplementär zu jeweils einem von mindestens zwei verschiedenen Zielgenen ist.

36. Verfahren nach einem der vorhergehenden Ansprüche, wobei eines der Zielgene das PKR-Gen ist.

37. Medikament mit mindestens einem Oligoribonukleotid mit doppelsträngiger Struktur (dsRNA) zur Hemmung der Expression eines vorgegebenen Zielgens, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist.

38. Medikament mit mindestens einem Vektor zur Kodierung mindestens eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Hemmung der Expression eines vorgegebenen Zielgens, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist.

39. Medikament nach Anspruch 37 oder 38, wobei die dsRNA oder der Vektor verpackt in micellare Strukturen, vorzugsweise in Liposomen, vorliegt.

40. Medikament nach Anspruch 37 oder 38, wobei die dsRNA oder der Vektor in virale natürliche Kapside oder in auf chemischem oder enzymatischem Weg hergestellte künstliche

Kapside oder davon abgeleitete Strukturen eingeschlossen ist.

41. Medikament nach einem der Ansprüche 37 bis 40, wobei dsRNA 10 bis 1000, vorzugsweise 15 bis 49, Basenpaare aufweist.

42. Medikament nach einem der Ansprüche 37 bis 41, wobei das Zielgen in eukaryontischen Zellen exprimierbar ist.

43. Medikament nach einem der Ansprüche 37 bis 42, wobei das Zielgen aus der folgenden Gruppe ausgewählt ist: Onkogen, Cytokin-Gen, Id-Protein-Gen, Entwicklungsgen, Priongen.

44. Medikament nach einem der Ansprüche 37 bis 43, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimierbar ist.

45. Medikament nach einem der Ansprüche 37 bis 44, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

46. Medikament nach Anspruch 45, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

47. Medikament nach Anspruch 45, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

48. Medikament nach einem der Ansprüche 37 bis 47, wobei die dsRNA abschnittsweise doppelsträngig ausgebildet ist.

49. Medikament nach einem der Ansprüche 37 bis 48, wobei der komplementäre Bereich I höchstens 49 aufeinanderfolgende Nukleotidpaare aufweist.

50. Medikament nach einem der Ansprüche 37 bis 49, wobei ein innerhalb der doppelsträngigen Struktur komplementärer Bereich II aus zwei separaten RNA-Einzelsträngen oder aus selbstkomplementären Bereichen eines, vorzugsweise zirkulär ausgebildeten, topologisch geschlossenen, RNA-Einzelstrangs gebildet ist.

51. Medikament nach einem der Ansprüche 37 bis 50, wobei der komplementäre Bereich II aus selbstkomplementären Bereichen einer RNA-Haarnadelschleife gebildet ist.

52. Medikament nach einem der Ansprüche 37 bis 51, wobei die Nukleotide im Schleifenbereich zwischen der doppelsträngigen Struktur zum Schutz vor Abbau chemisch modifiziert ist.

53. Medikament nach einem der Ansprüche 37 bis 52, wobei die Enden der dsRNA modifiziert sind, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

54. Medikament nach einem der Ansprüche 37 bis 53, wobei der durch die Nukleotidpaare bewirkte Zusammenhalt des komplementären Bereichs II durch mindestens eine, vorzugsweise zwei, weitere chemische Verknüpfung/en erhöht ist.

55. Medikament nach einem der Ansprüche 37 bis 54, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stapelungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet ist.

56. Medikament nach einem der Ansprüche 37 bis 55, wobei die chemische Verknüpfung an mindestens einem, vorzugsweise an beiden, Enden des komplementären Bereichs II hergestellt ist.

57. Medikament nach einem der Ansprüche 37 bis 56, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet ist, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Polyethylenglycol-Ketten sind.

58. Medikament nach einem der Ansprüche 37 bis 57, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Purinen benutzte Purinanaloga gebildet ist.

59. Medikament nach einem der Ansprüche 37 bis 58, wobei die chemische Verknüpfung durch in die komplementären Bereiche II eingeschaltete Azabenzoleinheiten gebildet ist.

60. Medikament nach einem der Ansprüche 37 bis 59, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet ist.

61. Medikament nach einem der Ansprüche 37 bis 60, wobei zur Herstellung der chemischen Verknüpfung mindestenes eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N`-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

62. Medikament nach einem der Ansprüche 37 bis 61, wobei die chemische Verknüpfung durch an den Enden des doppelsträn-

gigen Bereichs vorgesehene Thiophosphoryl-Gruppen gebildet ist.

63. Medikament nach einem der Ansprüche 37 bis 62, wobei die chemische Verknüpfung an den Enden des doppelsträngigen Bereichs vorgesehene Tripelhelix-Bindungen sind.

64. Medikament nach einem der Ansprüche 37 bis 63, wobei mindestens eine 2´-Hydroxylgruppe der Nukleotide der dsRNA in dem komplementären Bereich II durch eine chemische Gruppe, vorzugsweise eine 2´-Amino- oder eine 2´-Methylgruppe, ersetzt ist.

65. Medikament nach einem der Ansprüche 37 bis 64, wobei mindestens ein Nukleotid in mindestens einem Strang des komplementären Bereichs II ein "locked nucleotide" mit einem, vorzugsweise durch eine 2'-O, 4'-C-Methylenbrücke, chemisch modifizierten Zuckerring ist.

66. Medikament nach einem der Ansprüche 37 bis 65, wobei die dsRNA oder der Vektor an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben ist.

67. Medikament nach einem der Ansprüche 37 bis 66, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

68. Medikament nach einem der Ansprüche 37 bis 67, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

69. Medikament nach einem der Ansprüche 37 bis 68, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem

Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

70. Medikament nach einem der Ansprüche 37 bis 69, wobei die dsRNA zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

71. Medikament nach einem der Ansprüche 37 bis 70, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

72. Medikament nach einem der Ansprüche 37 bis 71, wobei darin mindestens zwei voneinander verschiedene dsRNAs oder mindestens ein dafür kodierender Vektor enthalten sind, wobei ein Strang jeder dsRNA zumindest abschnittsweise komplementär zu jeweils einem von mindestens zwei verschiedenen Zielgenen ist.

73. Medikament nach Anspruch 72, wobei eines der Zielgene das PKR-Gen ist.

74. Verwendung eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Herstellung eines Medikaments zur Hemmung der Expression eines vorgegebenen Zielgens, wobei ein Strang der dsRNA einen zum Zielgen zumindest abschnittsweise komplementären Bereich I aufweist.

75. Verwendung eines Vektors zur Kodierung mindestens eines Oligoribonukleotids mit doppelsträngiger Struktur (dsRNA) zur Herstellung eines Medikaments zur Hemmung der Expression eines vorgegebenen Zielgens, wobei ein Strang der dsRNA einen zu diesem Zielgen zumindest abschnittsweise komplementären Bereich I aufweist.

76. Verwendung nach Anspruch 74 oder 75, wobei die dsRNA oder der Vektor verpackt in micellare Strukturen, vorzugsweise in Liposomen, vorliegt.

77. Verwendung nach Anspruch 74 oder 75, wobei die dsRNA oder der Vektor in virale natürliche Kapside oder in auf chemischem oder enzymatischem Weg hergestellte künstliche Kapside oder davon abgeleitete Strukturen eingeschlossen ist.

78. Verwendung nach einem der Ansprüche 74 bis 77, wobei dsRNA 10 bis 1000, vorzugsweise 15 bis 49, Basenpaare aufweist.

79. Verwendung nach einem der Ansprüche 74 bis 78, wobei das Zielgen in eukaryontischen Zellen exprimierbar ist.

80. Verwendung nach einem der Ansprüche 74 bis 79, wobei das Zielgen aus der folgenden Gruppe ausgewählt ist: Onkogen, Cytokin-Gen, Id-Protein-Gen, Entwicklungsgen, Priongen.

81. Verwendung nach einem der Ansprüche 74 bis 80, wobei das Zielgen in pathogenen Organismen, vorzugsweise in Plasmodien, exprimierbar ist.

82. Verwendung nach einem der Ansprüche 74 bis 81, wobei das Zielgen Bestandteil eines Virus oder Viroids ist.

83. Verwendung nach Anspruch 82, wobei das Virus ein humanpathogenes Virus oder Viroid ist.

84. Verwendung nach Anspruch 82, wobei das Virus oder Viroid ein tier- oder pflanzenpathogenes Virus oder Viroid ist.

85. Verwendung nach einem der Ansprüche 74 bis 84, wobei die dsRNA abschnittsweise doppelsträngig ausgebildet ist.

86. Verwendung nach einem der Ansprüche 74 bis 85, wobei ein innerhalb der doppelsträngigen Struktur komplementärer Bereich II aus zwei separaten RNA-Einzelsträngen oder aus selbstkomplementären Bereichen eines, vorzugsweise zirkulär ausgebildeten, topologisch geschlossenen RNA-Einzelstrangs gebildet ist.

87. Verwendung nach einem der Ansprüche 74 bis 86, wobei der komplementäre Bereich II aus selbstkomplementären Bereichen einer RNA-Haarnadelschleife gebildet wird.

88. Verwendung nach einem der Ansprüche 74 bis 87, wobei die Nukleotide im Schleifenbereich zwischen der doppelsträngigen Struktur zum Schutz vor Abbau chemisch modifiziert sind.

89. Verwendung nach einem der Ansprüche 74 bis 88, wobei die Enden der dsRNA modifiziert sind, um einem Abbau in der Zelle oder einer Dissoziation in die Einzelstränge entgegenzuwirken.

90. Verwendung nach einem der Ansprüche 74 bis 89, wobei der durch die Nukleotidpaare bewirkte Zusammenhalt des komplementären Bereichs II durch mindestens eine, vorzugsweise zwei, weitere chemische Verknüpfung/en erhöht ist.

91. Verwendung nach einem der Ansprüche 74 bis 90, wobei die chemische Verknüpfung durch eine kovalente oder ionische Bindung, eine Wasserstoffbrückenbindung, hydrophobe Wechselwirkungen, vorzugsweise van-der-Waals- oder Stape-

lungswechselwirkungen, oder durch Metall-Ionenkoordination gebildet ist.

92. Verwendung nach einem der Ansprüche 74 bis 91, wobei die chemische Verknüpfung an mindestens einem, vorzugsweise an beiden, Enden des komplementären Bereichs II hergestellt ist.

93. Verwendung nach einem der Ansprüche 74 bis 92, wobei die chemische Verknüpfung mittels einer oder mehrerer Verbindungsgruppen gebildet ist, wobei die Verbindungsgruppen vorzugsweise Poly-(oxyphosphinicooxy-1,3-propandiol)- und/oder Polyethylenglycol-Ketten sind.

94. Verwendung nach einem der Ansprüche 74 bis 93, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Purinen benutzte Purinanaloga gebildet ist.

95. Verwendung nach einem der Ansprüche 74 bis 94, wobei die chemische Verknüpfung durch in den komplementären Bereichen II eingeführte Azabenzoleinheiten gebildet ist.

96. Verwendung nach einem der Ansprüche 74 bis 95, wobei die chemische Verknüpfung durch in den komplementären Bereichen II anstelle von Nukleotiden benutzte verzweigte Nukleotidanaloga gebildet ist.

97. Verwendung nach einem der Ansprüche 74 bis 96, wobei zur Herstellung der chemischen Verknüpfung mindestenes eine der folgenden Gruppen benutzt wird: Methylenblau; bifunktionelle Gruppen, vorzugsweise Bis-(2-chlorethyl)-amin; N-acetyl-N`-(p-glyoxyl-benzoyl)-cystamin; 4-Thiouracil; Psoralen.

98. Verwendung nach einem der Ansprüche 74 bis 97, wobei die chemische Verknüpfung durch an den Enden des doppelsträngigen Bereichs angebrachte Thiophosphoryl-Gruppen gebildet ist.

99. Verwendung nach einem der Ansprüche 74 bis 98, wobei die chemische Verknüpfung an den Enden des doppelsträngigen Bereichs durch Tripelhelix-Bindungen hergestellt ist.

100. Verwendung nach einem der Ansprüche 74 bis 99, wobei mindestens eine 2´-Hydroxylgruppe der Nukleotide der dsRNA in dem komplementären Bereich II durch eine chemische Gruppe, vorzugsweise eine 2´-Amino- oder eine 2´-Methylgruppe, ersetzt ist.

101. Verwendung nach einem der Ansprüche 74 bis 100, wobei mindestens ein Nukleotid in mindestens einem Strang des komplementären Bereichs II ein "locked nucleotide" mit einem, vorzugsweise durch eine 2'-O, 4'-C-Methylenbrücke, chemisch modifizierten Zuckerring ist.

102. Verwendung nach einem der Ansprüche 74 bis 101, wobei die dsRNA oder der Vektor an mindestens ein von einem Virus stammendes, davon abgeleitetes oder ein synthetisch hergestelltes virales Hüllprotein gebunden, damit assoziiert oder davon umgeben ist.

103. Verwendung nach einem der Ansprüche 74 bis 102, wobei das Hüllprotein vom Polyomavirus abgeleitet ist.

104. Verwendung nach einem der Ansprüche 74 bis 103, wobei das Hüllprotein das Virus-Protein 1 (VP1) und/oder das Virus-Protein 2 (VP2) des Polyomavirus enthält.

105. Verwendung nach einem der Ansprüche 74 bis 104, wobei bei Bildung eines Kapsids oder kapsidartigen Gebildes aus dem Hüllprotein die eine Seite zum Inneren des Kapsids oder kapsidartigen Gebildes gewandt ist.

106. Verwendung nach einem der Ansprüche 74 bis 105, wobei die dsRNA zum primären oder prozessierten RNA-Transkript des Zielgens komplementär ist.

107. Verwendung nach einem der Ansprüche 74 bis 106, wobei die Zelle eine Vertebratenzelle oder eine menschliche Zelle ist.

108. Verwendung nach einem der Ansprüche 74 bis 107, wobei mindestens zwei voneinander verschiedene dsRNAs oder mindestens ein dafür kodierender Vektor verwendet werden, wobei ein Strang jeder dsRNA zumindest abschnittsweise komplementär zu jeweils einem von mindestens zwei verschiedenen Zielgenen ist.

109. Verfahren nach Anspruch 108, wobei eines der Zielgene das PKR-Gen ist.

110. Verwendung nach einem der Ansprüche 74 bis 109, wobei das Medikament in die Blutbahn oder das Interstitium des zu therapierenden Organismus injizierbar ist.

111. Verwendung nach einem der Ansprüche 74 bis 110, wobei die dsRNA bzw. der sie kodierende Vektor in Bakterien oder Mikroorganismen aufgenommen sind.

112. Verwendung nach einem der Ansprüche 74 bis 111, wobei der komplementäre Bereich I höchstens 49 aufeinanderfolgende Nukleotidpaare aufweist.

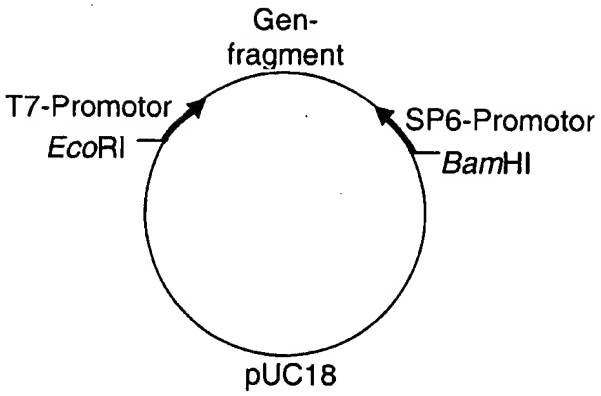


Fig. 1

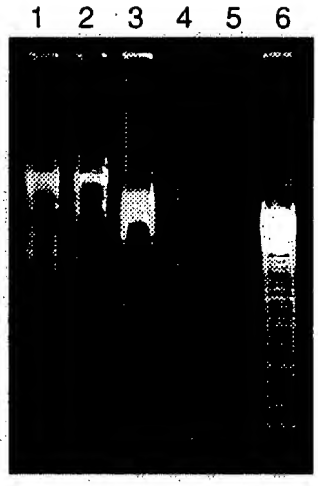


Fig. 2

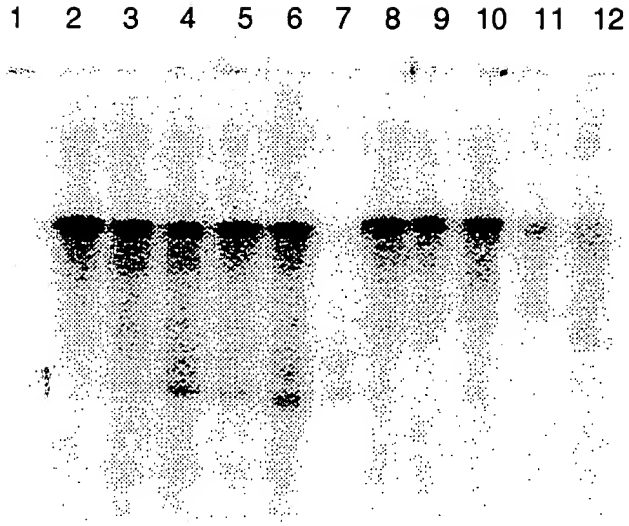


Fig. 3

Fig. 4 a

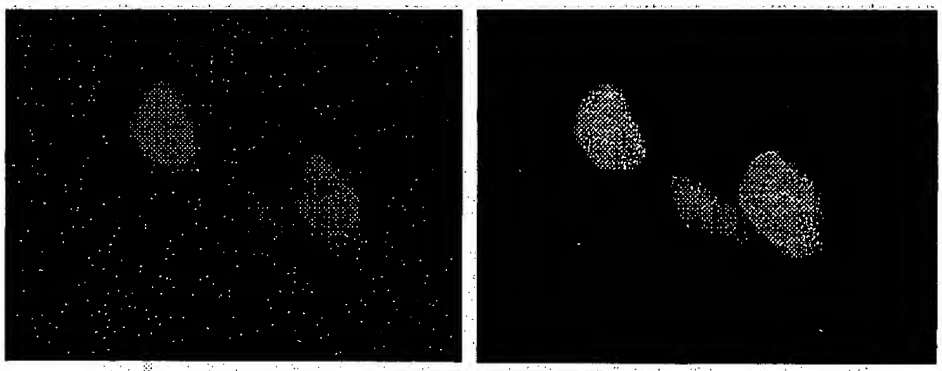


Fig. 4 b

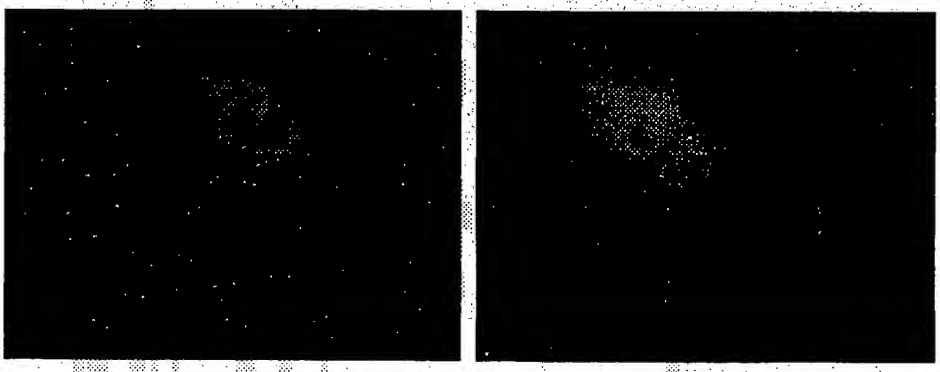


Fig. 4 c

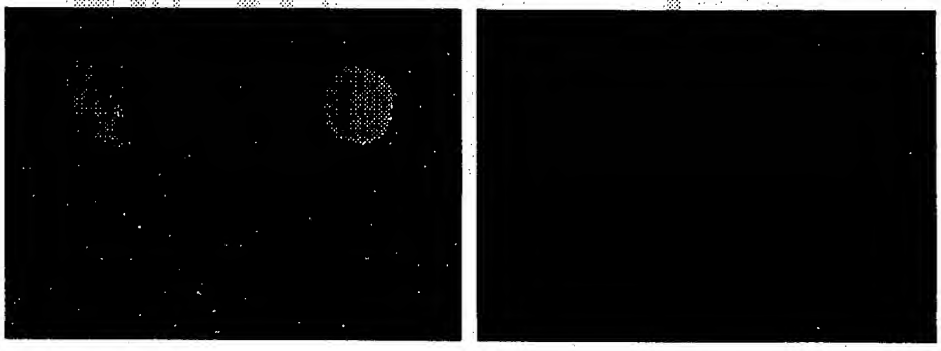


Fig. 4 d

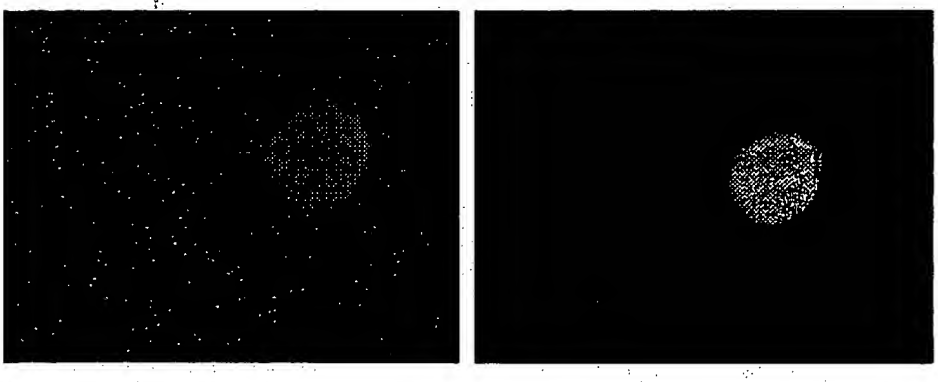
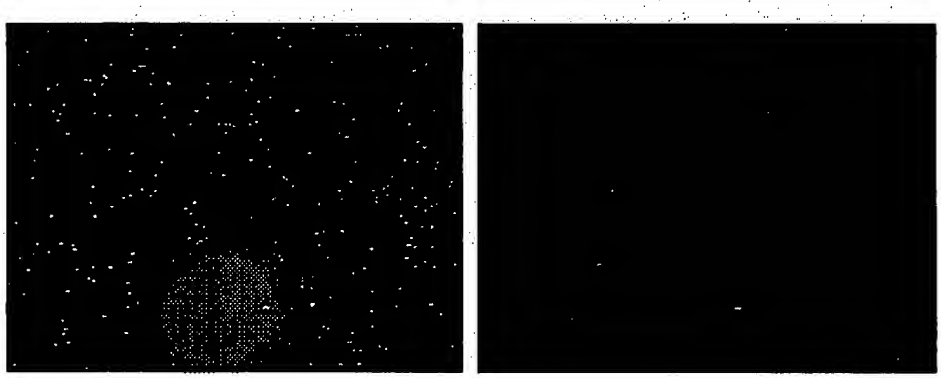


Fig. 4 e



SEQUENZPROTOKOLL

<110> Kreutzer Dr., Roland
Limmer Dr., Stephan

<120> Verfahren und Medikament zur Hemmung der Expression eines vorgegebenen Gens

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BamHI-Schnittstelle, SP6-RNA-Polymerasepromotor,
komplementärer Bereich zum YFP-Gen

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/DE 00/00244

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K31/713

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 19732 A (GENSET) 12 November 1992 (1992-11-12)	1-29, 32-34, 37-43, 45-66, 69-71, 74-80, 82-102, 105-108, 112
Y	abstract, page 11 lines 18-28 pages 12-13, page 15 line 22 bis page 20 line 1, pages 33 and 46, figures 1-6	1-35, 37-43, 45-72, 74-80, 82-108, 110-112

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2000	20/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gore, V

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/DE 00/00244

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 05770 A (ROTHBARTH KARSTEN ;JOSWIG GABY (DE); WERNER DIETER (DE); SCHUBERT) 12 February 1998 (1998-02-12)	1-29, 32-34, 37-43, 45-66, 69-71, 74-80, 82-102, 105-108, 112
Y	abstract, pages 2-3	1-35, 37-43, 45-72, 74-80, 82-108, 110-112
X,P	WO 99 32619 A (CARNEGIE INST OF WASHINGTON ;MONTGOMERY MARY K (US); FIRE ANDREW ()) 1 July 1999 (1999-07-01)	1-29, 32-34, 37-43, 45-66, 69-71, 74-80, 82-102, 105-108, 112
	abstract, pages 6, 11-12, 15-17	
Y	UHLMANN E ET AL: "ANTISENSE OLIGONUCLEOTIDES: A NEW THERAPEUTIC PRINCIPLE" CHEMICAL REVIEWS,US,AMERICAN CHEMICAL SOCIETY. EASTON, vol. 90, no. 4, 1 June 1990 (1990-06-01), pages 543-584, XP000141412 ISSN: 0009-2665	15-28, 52-65, 88-101
	pages 558, 565-566, 574-575	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/DE 00/00244

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INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
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A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
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Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 92 19732 A (GENSET) 12. November 1992 (1992-11-12)	1-29, 32-34, 37-43, 45-66, 69-71, 74-80, 82-102, 105-108, 112
Y	* Zusammenfassung, Seite 11 Z.18-28, Seiten 12-13, Seite 15 Z.22 bis Seite 20 Z.1, Seiten 33 und 46, Abbildungen 1-6 *	1-35, 37-43, 45-72, 74-80, 82-108, 110-112

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Int. Jonales Aktenzeichen
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C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
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X,P	WO 99 32619 A (CARNEGIE INST OF WASHINGTON ;MONTGOMERY MARY K (US); FIRE ANDREW ()) 1. Juli 1999 (1999-07-01) * Zusammenfassung, Seiten 6,11-12,15-17 *	1-29, 32-34, 37-43, 45-66, 69-71, 74-80, 82-102, 105-108, 112
Y	---	15-28, 52-65, 88-101
A	---	1-112

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Bd. 62, Dezember 1998 (1998-12), Seiten 1415-1434, XP000909741
Seiten 1422-1423 und 1428

2

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Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Inte onales Aktenzeichen
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EXHIBIT 12

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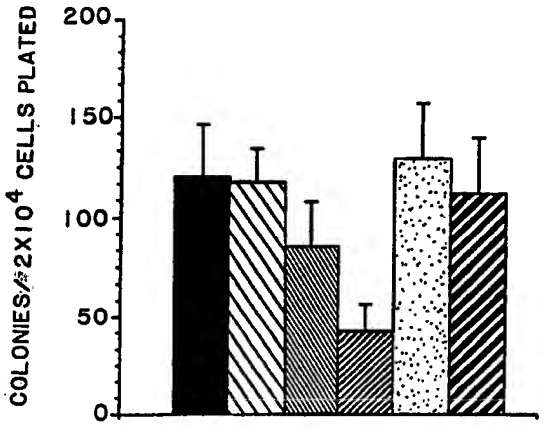
(54) Title: ANTISENSE OLIGONUCLEOTIDES TO C-KIT PROTO-ONCOGENE AND USES THEREOF



(57) Abstract

Oligonucleotides are provided having a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-*kit* gene. These "antisense" oligonucleotides are hybridizable to the c-*kit* mRNA transcript. Such oligonucleotides are useful in selectively inhibiting proliferation of erythroid cells, particularly in disorders characterized by an elevated hematocrit due to overproduction of erythrocytes. The antisense oligomers also have activity agent hematologic neoplastic cells and are therefore suitable as bone marrow purging agents.

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ANTISENSE OLIGONUCLEOTIDES TO C-KIT PROTO-ONCOGENE AND USES THEREOF

Field of the Invention

The invention relates to antisense oligonucleotides to proto-oncogenes, in particular to antisense oligonucleotides to the c-kit gene, and the use of such oligonucleotides to selectively inhibit proliferation of certain cells.

Reference to Government Grant

The invention described herein was supported in part by National Institutes of Health grants CA36896 and CA01324. The United States government has certain rights in the invention.

Background of the Invention

The c-kit gene is the normal homologue of v-kit, the HZ4 feline sarcoma virus oncogene. It resides on human chromosome 4. The gene encodes a dimeric transmembrane glycoprotein receptor with tyrosine kinase activity that appears to be highly related to the receptors for colony stimulating factor-1 and platelet derived growth factor. (Yarden et. al., The EMBO Journal, 6, 3341-3351 (1987)). Like these receptors, c-kit also appears to belong to the immunoglobulin gene superfamily.

The mouse c-kit gene has been mapped to chromosome 5 where it was determined to be allelic with the dominant white spotting locus (W) (Chabot et al., Nature 335, 88-89 (1988). C-kit mutations are commonly found in W mice and, in addition to abnormalities affecting coat color and gonadal development, they also have a variety of hematopoietic defects. Macrocytic anemia is one of the most striking and profound of these abnormalities. The W^{42} mutation, associated with a particularly severe hematologic manifestation, has been shown to be due to a missense mutation leading to replacement of one amino acid and defective tyrosine kinase activity (Tan et al., Science 247, 209 (1990)). Such animals are also known to have about one-third of the erythroid burst forming units of healthy wild-type littermates (Goldwather et al., Exp. Heme. 18, 936 (1990)).

The ligand for the c-kit receptor has now been identified, molecularly cloned and expressed (Yarden et. al., The EMBO Journal, 6, 3341-3351 (1987)). The encoded protein, known as stem cell factor (SCF), mast cell growth factor (MGF), or steel factor (SLF) is the product of a gene which resides at the steel (Sl) locus. Mice with Sl mutations have phenotypic abnormalities quite similar to those of W mice. The W mouse lacks, or has defects in, a critical signal transducing receptor encoded by c-kit. The Sl mouse has defects in the ligand which stimulates the receptor.

The importance of the c-kit ligand-receptor system in human hematopoiesis is unclear. No human mutations at the corresponding locii have been described. Studies in mice may have very limited applicability to human systems. Moreover, even if a tissue is shown to express a particular message, the importance of the message to expression of a cellular phenotype is not known until the cell is deprived of the encoded protein. Biological systems are redundant. Lack of a protein can often be compensated by another protein of the same fami-

ly. It is therefore not predictable that inhibition of expression of a particular gene will result in altered phenotype.

Summary of the Invention

Antisense oligonucleotides and pharmaceutical compositions thereof with pharmaceutical carriers are provided. Each oligonucleotide has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene. The oligonucleotide is hybridizable to the mRNA transcript. Preferably, the oligonucleotide is at least a 12-mer oligonucleotide, that is, an oligomer containing at least 12 nucleotide residues. In particular, the oligomer is advantageously a 12-mer to a 40-mer, preferably an oligodeoxynucleotide. While oligonucleotides smaller than 12-mers may be utilized, they are statistically more likely to hybridize with non-targeted sequences, and for this reason may be less specific. In addition, a single mismatch may destabilize the hybrid. While oligonucleotides larger than 40-mers may be utilized, uptake may be more difficult. Moreover, partial matching of long sequences may lead to non-specific hybridization, and non-specific effects. Preferably, the oligonucleotide is a 15- to 30-mer oligodeoxynucleotide, more advantageously an 18- to 26-mer. A 15- to 21-mer is most preferred.

While in principle oligonucleotides having a sequence complementary to any region of the c-kit gene find utility in the present invention, oligonucleotides complementary to a portion of the c-kit mRNA transcript including the translation initiation codon are particularly preferred. Also preferred are oligonucleotides complementary to a portion of the c-kit mRNA transcript lying within about 40 nucleotides upstream (the 5′ direction) or about 40 nucleotides downstream (the 3′ direction) from the translation initiation codon.

The invention also provides a method for inhibiting proliferation of erythroid cells comprising administering to a host in need of such treatment an effective amount of the c-kit antisense oligonucleotides of the invention.

The invention provides a method for treating hematologic neoplasms characterized by c-kit expression comprising administering an effective amount of c-kit antisense oligonucleotide in vivo or ex vivo to a host in need of such treatment, or to cells harvested from the host.

Administration of the c-kit oligonucleotides is also useful in treatment of malignant melanoma, and testicular or ovarian tumors.

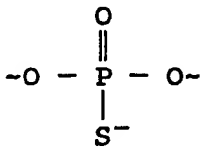
As used in the herein specification and appended claims, unless otherwise indicated, the term "oligonucleotide" includes both oligomers of ribonucleotide i.e., oligoribonucleotides, and oligomers of deoxyribonucleotide i.e., oligodeoxyribonucleotides (also referred to herein as "oligodeoxynucleotides"). Oligodeoxynucleotides are preferred.

As used herein, unless otherwise indicated, the term "oligonucleotide" also includes oligomers which may be large enough to be termed "polynucleotides".

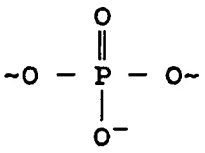
The terms "oligonucleotide" and "oligodeoxynucleotide" include not only oligomers and polymers of the biologically significant nucleotides, i.e. nucleotides of adenine ("A"), deoxyadenine ("dA"), guanine ("G"), deoxyguanine ("dG"), cytosine ("C"), deoxycytosine ("dC"), thymine ("T") and uracil ("U"), but also oligomers and polymers hybridizable to the c-kit mRNA transcript which may contain other nucleotides. Likewise, the terms "oligonucleotide" and "oligodeoxynucleotide" include oligomers and polymers wherein one or more purine or pyrimidine moieties, sugar moieties or internucleotide linkages is chemically modified. The term "oligonucleotide" is thus understood to also include oligomers which may prop-

erly be designated as "oligonucleosides" because of modification of the internucleotide phosphodiester bond. Such modified oligonucleotides include, for example, the alkylphosphonate oligonucleosides, discussed below.

The term "phosphorothioate oligonucleotide" means an oligonucleotide wherein one or more of the internucleotide linkages is a phosphorothioate group,

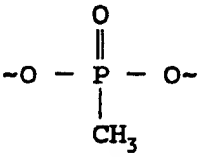


as opposed to the phosphodiester group



which is characteristic of unmodified oligonucleotides.

By "alkylphosphonate oligonucleoside" is meant an oligonucleotide wherein one or more of the internucleotide linkages is an alkylphosphonate group,



wherein R is an alkyl group, preferably methyl or ethyl.

The term "downstream" when used in reference to a direction along a nucleotide sequence means the 5′→3′ direction. Similarly, the term "upstream" means the 3′→5′ direction.

The term "c-kit mRNA transcript" means the presently known mRNA transcript of the human c-kit gene, or any further transcripts which may be elucidated.

Description of the Figures

Figure 1 is an autoradiograph of a reverse transcription-polymerase chain recaction gel indicating the increase in c-kit mRNA at intervals following stimulation of adherent, T lymphocyte-depleted human bone marrow cells (A^-T^-MNC) with 20 U/ml interleukin-3 and 5 U/ml erythropoietin: Lane 1 (t = 0); lane 2 (2 hrs); lane 3 (8 hrs); lane 4 (12 hrs); lane 5 (24 hrs); lane 6 (36 hrs); lane 7 (48 hrs); lane 8 (H_2O control).

Figure 2 is a similar autoradiograph indicating the effect of c-kit oligomer exposure on c-kit mRNA levels in A^-T^-MNC after stimulation by interleukin-3 and erythropoietin in 5% AB serum. Lane 1 (no oligomer, t = 0); lane 2 (no oligomer, t = 36 hrs); lane 3 (sense oligomer, 36 hrs); lane 4 (antisense, 36 hrs); lane 5 (scrambled sequence with identical base content, 36 hrs).

Figure 3 shows the effect of c-kit oligodeoxyynucleotides on BFU-E-derived colony formation. Oligomers were added to cultures at time zero, and 50% of the initial dose was again added 18 hours later. The bars on the graph indicate: 1, untreated control cells; 2, antisense treatment of 20 μg/ml followed by 10 μg/ml; 3, antisense treatment of 40 μg/ml followed by 20 μg/ml; 4, antisense treatment of 100 μg/ml followed by 50 μg/ml; 5, sense treatment of 100 μg/ml followed by 50 μg/ml; 6, scrambled-sequence treatment of 100 μg/ml followed by 50 μg/ml.

Detailed Description of the Invention

We have discovered that the c-kit gene is of predominant importance in human erythropoiesis. We have found that the protein which this gene expresses, a receptor for tyrosine kinase, transduces a signal which acts in concert with interleukin-3 (IL-3) to optimize cell proliferation, particularly erythroid burst forming units (BFU-E).

The putative DNA sequence complementary to the mRNA transcript of the human c-kit gene has been reported by Yarden et al., The EMBO Journal, 6, 3341-3351 (1987), the entire disclosure of which is incorporated herein by reference. The nucleotide sequence and predicted amino acid sequence are set forth in Figure 3 thereof. The c-kit polypeptide is synthesized by translation of a single 5-kb mRNA, which contains an open reading frame coding for a 976 amino acid polypeptide.

The antisense oligonucleotides of the invention may be synthesized by any of the known chemical oligonucleotide synthesis methods. Such methods are generally described, for example, in Winnacker, From Genes to Clones: Introduction to Gene Technology, VCH Verlagsgesellschaft mbH (H. Ibelgaufts trans. 1987).

Any of the known methods of oligonucleotide synthesis may be utilized in preparing the instant antisense oligonucleotides.

The antisense oligonucleotides are most advantageously prepared by utilizing any of the commercially available, automated nucleic acid synthesizers, for example, the Applied Biosystems 380B DNA Synthesizer, which utilizes β-cyanoethyl phosphoramidite chemistry.

Since the complete nucleotide synthesis of DNA complementary to the c-kit mRNA transcript is known, antisense oligonucleotides hybridizable with any portion of the mRNA transcript may be prepared by the oligonucleotide synthesis methods known to those skilled in the art.

While any length oligonucleotide may be utilized in the practice of the invention, sequences shorter than 12 nucleotides may be less specific in hybridizing to the target c-kit mRNA, may be more easily destroyed by enzymatic digestion, and may be destabilized by even a single base pair mismatch. Hence, oligo-

nucleotides having 12 or more nucleotides are preferred.

Long sequences, particularly sequences longer than about 40 nucleotides, may be somewhat less effective in inhibiting c-kit translation because of decreased uptake by the target cell. Thus, oligomers of 12-40 nucleotides are preferred, more preferably 15-30 nucleotides, most preferably 18-26 nucleotides. Sequences of 18-21 nucleotides are particularly preferred. While sequences of 18-21 nucleotides are most particularly preferred, for unmodified oligonucleotides, slightly longer chains of up to about 26 nucleotides, are preferred for modified oligonucleotides such as phosphorothioate oligonucleotides, which hybridize less strongly to mRNA than unmodified oligonucleotides.

Oligonucleotides complementary to and hybridizable with any portion of the c-kit mRNA transcript are, in principle, effective for inhibiting translation of the transcript, and capable of inducing the effects herein described. It is believed that translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the 5'-terminal region of the c-kit mRNA transcript are preferred. The oligonucleotide is preferably directed to a site at or near the initiation codon for protein synthesis. The following 40-mer oligodeoxynucleotide is complementary to the c-kit mRNA transcript beginning with the initiation codon of the transcript and extending downstream (in the 5' direction): GAACGCAGAG AAAATCCCAG GCGCCGCGAG CGCCTCTCAT (SEQ ID NO:1).

Smaller oligomers based upon the above sequence, in particular oligomers hybridizable to segments of the c-kit message containing the initian codon, may be utilized. Particularly preferred are the following 15- to 26-mers:

(SEQ ID NO:2)

(SEQ ID NO:3)
(SEQ ID NO:4)
(SEQ ID NO:5)
(SEQ ID NO:6)
(SEQ ID NO:7)
(SEQ ID NO:8)
(SEQ ID NO:9)
(SEQ ID NO:10)
(SEQ ID NO:11)
(SEQ ID NO:12)
(SEQ ID NO:13)

Oligonucleotides hybridizable to the c-kit mRNA transcript finding utility according to the present invention include not only native oligomers of the biologically significant nucleotides, i.e., A, dA, G, dG, C, dC, T and U, but also oligonucleotide species which have been modified for improved stability and/or lipid solubility. The oligonucleotides may be any of a number of types, including those having a charged or uncharged backbone. For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or sulfur atom for a phosphate oxygen in the internucleotide phosphodiester linkage to form alkylphosphonate oligonucleotide or phosphorothioate oligonucleotides. The phosphorothioates, in particular, are stable to nuclease cleavage and soluble in lipid. They may be synthesized by known automatic synthesis methods.

The oligonucleotide employed may represent an unmodified oligonucleotide or an oligonucleotide analog. One group of such analogs, the alkyl phosphonates, includes but is not limited to the ethyl or methyl phosphonate analogs disclosed by U.S. Patent No. 4,469,863.

Non-ionic oligonucleotides are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability

to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in U.S. Patent 4,469,863.

Methylphosphonate oligomers can be prepared by a variety of methods, both in solution and on insoluble polymer supports (Agrawal and Riftina, Nucl. Acids Res., 6, 3009-3024 (1979); Miller et al., Biochemistry, 18, 5134-5142 (1979), Miller et al., J. Biol. Chem., 255, 9659-9665 (1980); Miller et al., Nucl. Acids Res., 11, 5189-5204 (1983), Miller et al., Nucl. Acids Res., 11, 6225-6242 (1983), Miller et al., Biochemistry, 25, 5092-5097 (1986); Engels and Jager, Angew. Chem. Suppl. 912 (1982); Sinha et al., Tetrahedron Lett. 24, 877-880 (1983); Dorman et al, Tetrahedron, 40, 95-102 (1984); Jager and Engels, Tetrahedron Lett., 25, 1437-1440 (1984); Noble et al., Nucl. Acids Res., 12, 3387-3404 (1984); Callahan et al., Proc. Natl. Acad. Sci. USA, 83, 1617-1621 (1986); Koziolkiewicz et al., Chemica Scripta, 26, 251-260 (1986); Agrawal and Goodchild, Tetrahedron Lett., 38, 3539-3542 (1987); Lesnikowski et al., Tetrahedron Lett., 28, 5535-5538 (1987); Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

The most efficient procedure for preparation of methylphosphonate oligonucleosides involves use of 5'-O-dimethoxytrityldeoxynucleoside-3'-O-diisopropylmethylphosphoramidite intermediates, which are similar to the methoxy or β-cyanoethyl phosphoramidite reagents used to prepare oligodeoxyribonucleotides. The methylphosphonate oligomers can be prepared on controlled pore glass polymer supports using anautomated DNA synthesizer (Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoroamidites ac-

cording to the procedure of Dagle et al., Nucl. Acids Res. 18, 4751-4757 (1990).

Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the property of cellular uptake via a receptor (Loke et al., Proc. Natl. Acad. Sci. U.S.A. 86, 3474-3478 (1989)).

Phosphorothioate oligodeoxynucleotide are described by LaPlanche, et al., Nucleic Acids Research 14, 9081 (1986) and by Stec et al., J. Am. Chem. Soc. 106, 6077 (1984). The general synthetic method for phosphorothioate oligonucleotides was modified by Stein et al., Nucl. Acids Res., 16, 3209-3221 (1988), so that these compounds may readily be synthesized on an automatic synthesizer using the phosphoramidite approach.

Furthermore, recent advances in the production of oligoribonucleotide analogues mean that other agents may also be used for the purposes described here, e.g., 2'-O-methylribonucleotides (Inoue et al., Nucleic Acids Res. 15, 6131 (1987) and chimeric oligonucleotides that are composite RNA-DNA analogues (Inoue et al., FEBS Lett. 215, 327 (1987).

While inhibition of c-kit mRNA translation is possible utilizing either antisense oligoribonucleotides or oligodeoxyribonucleotides, free oligoribonucleotides are more susceptible to enzymatic attack by ribonucleases than oligodeoxyribonucleotides. Hence, oligodeoxyribonucleotides are preferred in the practice of the present invention. Oligodeoxyribonucleotides are further preferred because, upon hybridization with c-kit mRNA, the resulting DNA-RNA hybrid duplex is a substrate for RNase H, which specifically attacks the

RNA portion of DNA-RNA hybrid. Degradation of the mRNA strand of the duplex releases the antisense oligodeoxynucleotide for hybridization with additional c-kit messages.

In general, the antisense oligonucleotides of the present invention will have a sequence which is completely complementary to the target portion of the c-kit message. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "nucleotide sequence complementary to at least a portion of the mRNA transcript" of c-kit does not necessarily mean a sequence having 100% complementarity with the transcript. In general, any oligonucleotide having sufficient complementarity to form a stable duplex with c-kit mRNA, that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target region of the c-kit message. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. More than one mismatch probably will not be tolerated for antisense oligomers of less than about 21 nucleotides. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any given antisense oligomer and the target c-kit message sequence, based upon the melting point, and therefore the stability of the resulting duplex. Melting points of duplexes of a given base pair composition can be determined from standard texts, such as Molecular Cloning: A Laboratory Manual, (2nd edition, 1989), J. Sambrook et al., eds.

While oligonucleotides capable of stable hybridization with any region of the c-kit message are within the scope of the present invention, oligonucleotides complementary to a region including the initiation codon are believed particularly effective. Par-

ticularly preferred are oligonucleotides hybridizable to a region of the c-kit mRNA up to 40 nucleotides upstream (in the 5' direction) of the initiation codon or up to 40 nucleotides downstream (in the 3' direction) of that codon.

The antisense oligonucleotides of the invention inhibit human erythropoiesis, as indicated by inhibition of colony forming unit-erythroid cells (CFU-E) and burst forming unit-erythroyd cells (BFU-E). However, they do not appear to inhibit proliferation of cells of other lineages, such as colony forming unit-granulocyte-macrophage cells (CFU-GM) and colony forming unit-megakaryocyte cells (CFU-MEG). CFU-GM cells and CFU-MEG cells are the progenitors of blood granulocytes and platelets, respectively. This pharmaceutically significant differential sensitivity makes the instant oligonucleotides useful in treating disorders characterized by elevated production of red blood cells.

The antisense oligonucleotides of the invention are believed useful in the treatment of any one of a variety of conditions characterized by an elevated hematocrit due to overproduction of erythrocytes. One such disorder, polycythemia, may arise from a variety of causes and is classified as either relative, secondary or primary polycythemia.

In relative polycythemia, the red cell mass is normal. Plasma volume is decreased. The increase in erythrocytes is therefore a concentration effect. Relative polycythemia is associated with diabetic acidosis, diarrhea, or diabetes insipidus. It is also associated with the intake of diuretics.

In secondary polycythemia, red cell mass is increased secondarily to elevated erythropoietin (EPO) production. This occurs in individuals who have located to higher altitudes, since decreased oxygen simulates anemia, which is a triggering signal for increase

of erythrocyte production. Secondary polycythemia may also occur in patients who have significant pulmonary or cardiac disfunction. Decreased oxygen delivery to tissues simulates anemia which triggers a signal to increase erythrocyte production. Secondary polycythemia may also occur in individuals who have tumors which are capable of synthesizing EPO, as in hypernephroma, cerebellar hemangioma and uterine leiomyoma.

Primary polycythemia is characterized by an increase in red cell mass, with either normal or decreased EPO levels. Primary polycythemia occurs in the myeloproliferative disorders, in particular polycythemia vera (PV). Disorders such as PV are true stem cell disorders. Accordingly, the white blood cell count and platelet count may be elevated. However, control of erythrocyte production is the primary objective in management of PV. Control of PV is usually effected by phlebotomy in secondary causes (if treatment of the primary disease is ineffective), and by a combination of phlebotomy and chemotherapy. Chemotherapeutic treatment of PV typically utilizes alkylating agents such as busulfan, melphalan, cyclophosphamide, chlorambucil or radioactive phosphorous in the form of sodium phosphate-^{32}P.

The rapid fluid shifts imposed by phlebotomy in the treatment of PV can be dangerous for patients with cardiac/pulmonary disease. Phlebotomy is also associated with a significant risk of fatal thrombosis. (Burk et al., Semin. Hematol. 23, 132 (1986); Ellis et al., id. at 144). Control of erythrocyte production by administration of the c-kit antisense oligomers of the present invention is an attractive alternative to phlebotomy and chemotherapy.

The antisense oligonucleotides of the invention are further believed to possess utility in the treatment of hematologic malignancies. Hematologic neoplastic cells believed sensitive to the instant c-

kit antisense oligonucleotides include, for example, myeloid leukemia cells. The appearance of these cells in the bone marrow and elsewhere in the body is associated with various disease conditions, such as all of the various French-American-British (FAB) subtypes of acute myeloid leukemia, and chronic myeloid leukemia.

The c-kit antisense oligonucleotides are believed particularly useful against acute myelogenous leukemia (AML). Significant activity against chronic myelogenous leukemia (CML) has also been demonstrated. CML, in particular, is characterized by abnormal proliferation of immature granulocytes - neutrophils, eosinophils, and basophils - in the blood, the bone marrow, the spleen, the liver, and sometimes other tissues. The essential feature is accumulation of granulocytic precursors in these tissues. The patient who presents symptoms will characteristically have more than 20,000 white blood cells per μl, and the count may exceed 400,000. Virtually all CML patients will develop "blast crisis", the terminal stage of the disease during which immature blast cells rapidly proliferate, leading to patient death.

Since c-kit function appears to be important for development of melanocytes, i.e., neural crest-derived pigment cells, and germ (gonadal) cells, it is believed that the antisense oligonucleotides of the present invention are useful for the treatment of malignant melanoma and testicular or ovarian tumors.

The antisense oligonucleotides of the invention find utility as bone marrow purging agents. They may be utilized in vitro to cleanse bone marrow contaminated by hematologic neoplasms. They are believed useful as purging agents in either allogeneic or autologous bone marrow transplantation. They are believed particularly effective in the treatment of hematological malignancies or other neoplasias which metastasize in the bone marrow.

According to a method for bone marrow purging, bone marrow is harvested from a donor by standard operating room procedures from the iliac bones of the donor. Methods of aspirating bone marrow from donors are well-known in the art. Examples of apparatus and processes for aspirating bone marrow from donors are disclosed in U.S. Patents 4,481,946 and 4,486,188. Sufficient marrow is withdrawn so that the recipient, who is either the donor (autologous transplant) or another individual (allogeneic transplant), may receive from about 4 x 10^8 to about 8 x 10^8 processed marrow cells per kg of bodyweight. This generally requires aspiration of about 750 to about 1000 ml of marrow. The aspirated marrow is filtered until a single cell suspension, known to those skilled in the art as a "buffy coat" preparation, is obtained. This suspension of leukocytes is treated with c-kit antisense oligonucleotides in a suitable carrier, advantageously in a concentration of about 8 mg/ml. Alternatively, the leucocyte suspension may be stored in liquid nitrogen using standard procedures known to those skilled in the art until purging is carried out. The purged marrow can be stored frozen in liquid nitrogen until ready for use. Methods of freezing bone marrow and biological substances are disclosed, for example, in U.S. Patents 4,107,937 and 4,117,881.

Other methods of preparing bone marrow for treatment with c-kit antisense may be utilized, which methods may result in even more purified preparations of hematopoietic cells than the aforesaid buffy coat preparation.

One or more hematopoietic growth factors may be added to the aspirated marrow or buffy coat preparation to stimulate growth of hematopoietic neoplasms, and thereby increase their sensitivity to the toxicity of the c-kit antisense oligonucleotides. Such hematopoietic growth factors include, for example, IL-3 and

granulocyte macrophage colony stimulating factor (GM-CSF). The recombinant human versions of such growth factors are advantageously employed.

After treatment with the antisense oligonucleotides, the cells to be transferred are washed with autologous plasma or buffer to remove unincorporated oligomer. The washed cells are then infused back into the patient.

For in vivo use, the antisense oligonucleotides may be combined with a pharmaceutical carrier, such as a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solution of dextrose, and the like. For in vivo antineoplastic use and in vivo erythroid cell reduction, the c-kit mRNA antisense oligonucleotides are preferably administered parenterally, most preferably intravenously. The vehicle is designed accordingly. It is also possible to administer such compounds ex vivo by isolating lymphocytes from peripheral blood, treating them with the antisense oligonucleotides, then returning the treated lymphocytes to the peripheral blood of the donor. Ex vivo techniques have been utilized in treatment of cancer patients with interleukin-2 activated lymphocytes, and are well-known to those skilled in the art.

In addition to administration with conventional carriers, the antisense oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques. For example, oligonucleotides may be encapsulated in liposomes for therapeutic delivery. The oligonucleotide, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids

such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature. Oligonucleotides have been successfully encapsulated in unilameller liposomes.

Reconstituted Sendai virus envelopes have been successfully used to deliver RNA and DNA to cells. Arad et al., Biochem. Biophy. Acta. 859, 88-94 (1986).

The oligonucleotides may be conjugated to poly(L-lysine) to increase cell penetration. Such conjugates are described by Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652 (1987). The procedure requires that the 3'-terminal nucleotide be a ribonucleotide. The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and then reduced with sodium cyanoborohydride. This procedure converts the 3'-terminal ribose ring into a morpholine structure antisense oligomers.

For ex vivo antineoplastic application, such as, for example, in bone marrow purging, the c-kit antisense oligonucleotides may be administered in amounts effective to kill neoplastic cells while maintaining the viability of normal hematologic cells. Such amounts may vary depending on the nature and extent of the neoplasm, the particular oligonucleotide utilized, the relative sensitivity of the neoplasm to the oligonucleotide, and other factors. Concentrations from about 10 to 200 μg/ml per 10^5 cells may be employed, preferably from about 40 to 150 μg/ml per 10^5 cells. Supplemental dosing of the same or lesser amounts of oligonucleotide are advantageous to optimize the treatment. Thus, for purging bone marrow containing 2 x 10^7 cell per ml of marrow volume, dosages of from about 2 to 40 mg antisense per ml of marrow may be effectively utilized, preferably from about 8 to 24

mg/ml. Greater or lesser amounts of oligonucleotide may be employed.

For in vivo use, the c-kit antisense oligonucleotides may be administered in an amount sufficient to result in extracellular concentrations approximating the above stated in vitro concentrations. Preferably, the intracellular concentration is in the range of from about 10 to about 100 μg/ml. The actual dosage administered may take into account the size and weight of the patient, whether the nature of the treatment is prophylactic or therapeutic in nature, the age, weight, health and sex of the patient, the route of administration, and other factors. Those skilled in the art should be readily able to derive suitable dosages and schedules of administration to suit the specific circumstance. The daily dosage may range from about 0.1 to 1,000 mg oligonucleotide per day, preferably from about 10 to about 1,000 mg per day. Greater or lesser amounts of oligonucleotide may be administered, as required. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstances and needs of the patient.

The present invention is described in greater detail in the following non-limiting examples.

Example 1

Effect of c-kit Antisense Oligomer Exposure on Normal Hematopoietic Progenitor Cell Growth.

The effect of c-kit antisense oligonucleotide on hematopoietic progenitor cell cloning efficiency and development was systematically investigated by assessing CFU-E, burst-forming unit-erythroid (BFU-E), CFU-GM, and CFU-MEG growth after oligomer exposure.

Cells: Human bone marrow cells (BMC) were obtained from normal, healthy volunteers by Ficoll-

Hypaque density gradient centrifugation, and were partially enriched for hematopoietic progenitors by removal of adherent, phagocytic elements and T lymphocytes (Gewirtz et al., J. Immunol. 139, 2915-2925 (1987)). For some experiments, the adherent, T lymphocyte-depleted population (A^-T^-MNC) was further enriched by positively selecting $CD34^+$ cells with immunomagnetic beads (Dynal A.S., Oslo, Norway). The A^-T^-MNC cells were suspended in supplemented alpha medium and incubated with mouse anti-HPCA-I antibody in 1:20 dilution, 45 minutes, at 4°C with gentle inverting of tubes. Cells were washed x 3 in Supplemented alpha medium, and then incubated with beads coated with the Fc fragment of goat anti-mouse IgG_1 (75 μl of immunobeads/10^7 A^-T^- MNC). After 45 minutes of incubation (4°C), cells adherent to the beads were positively selected using a magnetic particle concentrator as directed by the manufacturer.

Oligodeoxynucleotides: Unmodified, 18-nucleotide oligodeoxynucleotides (oligomers) were synthesized as previously reported (Gewirtz et al., Science 242, 1303-1306 (1988)). In brief, oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer by means of a β-cyanoethyl phosphoramidite chemistry. Oligomers were purified by ethanol precipitation and multiple washes in 70% ethanol. They were then lyophilized to dryness and redissolved in culture medium prior to use at a concentration of 1μg/μl (0.175 μM). Oligomer sequences employed, corresponding to codons 1-6 of the published human c-kit cDNA sequence (Yarden, et al., The EMBO Journal 6, 3341-3351 (1987)), were as follows: ATGAGAGGCG CTCGCGGC (SEQ ID NO:14), sense oligomer; GCCGCGAGCG CCTCTCAT (SEQ ID NO:10), antisense oligomer; and GCACCGTCTG CCAGTCGC (SEQ ID NO:15), scrambled sequence oligomer.

Oligomer Treatment of Cells: Cells were exposed to oligomers as previously described (Gewirtz

et al., Science 242, 1303-1306 (1988)). 2 x 10^5 A^-T^-MNC or $CD34^+$MNC were incubated in 5 ml polypropylene tubes (Fisher Scientific, Pittsburgh, PA) in a total volume of 0.4 ml of Iscove's modified Dulbecco's medium (IMDM) containing 2% human AB serum and 10 mM Hepes buffer. Oligomers were added at time zero (2.5-100 μg/ml), and 50% of the initial dose was added again 18 hours later (final total concentration ~0.6-26 μM). Twenty-four hours after the first addition of oligomers, cells were prepared for plating in plasma clot or methylcellulose cultures. Cells (1 x 10^5 A^-T^-MNC or 1 x 10^4 $CD34^+$MNC per dish) were not washed before plating. Control cultures were manipulated in an identical manner but were not treated with oligomers.

Colony Assays: Assays for hematopoietic progenitor cells of varying lineages were carried out essentially as reported (Id.). In brief, cells (10^5 A^-T^-MNC or 5 x 10^3 $CD34^+$MNC) were resuspended in IMDM supplemented with 30% human AB serum, 1% BSA, 10^{-4} M mercaptoethanol, and 10% citrated bovine plasma (Hyclone Laboratories, Denver, CO). Addition of the appropriate recombinant human growth factors allowed for stimulation of the following cell types:

CFU-E: 5 U/ml EPO;
BFU-E: 20 U/ml IL-3 and 5 U/ml EPO, or 100 ng/ml SCF and 5 U/ml EPO;
CFU-GM: 20 U/ml IL-3 and 5 ng/ml granulocyte-macrophage colony stimulating factor;
CFU-MEG: 20 U/ml IL-3 and 100 ng/ml IL-6.

One ml volumes were cultured in 35 mm petri dishes at 37°C, 5% CO_2, and 95% humidity. CFU-E colonies were scored at day 7, BFU-E colonies at day 14, CFU-MEG at day 12, and CFU-GM at day 11 of incubation. Colony identification was carried out as previously described (Id.).

Statistics: Statistical significance of differences between means of test groups was assessed by Mann-Whitney non-parametric analysis using a statistical software package (Statview 512+, BrainPower, Inc., Calabasas, CA). The results appear in Tables 1 and 2. Values presented are actual colonies counted, pooled from two or three individual studies.

Table 1

Effect of c-kit on A^-T^- cell derived colony formation

Progenitor Cell Type	Control	Sense	Scrambled Sequence	Antisense
CFU-E	182, 209 1943, 543 148, 100 Mean (±SE)= 522±291	153, 142 1635, 1135 129, 176 Mean (±SE)= 562±268	119, 128 627, 649 149, 206 Mean (±SE)= 313±103	33, 59 243, 213 97, 107 Mean (±SE)= 125±34
BFU-E	133, 152 534, 392 206, 172 Mean (±SE)= 265±66	117, 106 601, 249 215, 258 Mean (±SE)= 258±74	94, 64 273, 246 162, 246 Mean (±SE)= 181±36	60, 149 126, 113 59, 51 Mean (±SE)= 76±14
CFU-GM	212, 189 412, 408 217, 241 209, 246 Mean (±SE)= 280±42	231, 179 395, 421 230, 237 Mean (±SE)= 282±41	282, 193 457, 384 201, 199 Mean (±SE)= 286±46	195, 220 407, 471 293, 187 Mean (±SE)= 296±49
CFU-MEG	114, 107 93, 100 Mean (±SE)= 104±5	133, 117 58, 52 Mean (±SE)= 90±20	154, 113 53, 40 Mean (±SE)= 90±27	127, 112 47, 54 Mean (±SE)= 85±20

Table 2

Effect of c-kit oligomers on $CD34^+$ dell derived colony formation

Progenitor Cell Type	Control	Sense	Scrambled Sequence	Antisense
CFU-E	5, 9 16, 14 71, 79 Mean (±SE)= 32±12	16, 14 11, 17 78, 48 Mean (±SE)= 31±11	6, 21 22, 8 86, 91 Mean (±SE)= 39±15	1, 0 0, 0 40, 22 Mean (±SE)= 11±7
BFU-E	179, 229 276, 281 271, 451 Mean (±SE)= 281±37	293, 120 241, 151 440, 361 Mean (±SE)= 286±50	191, 261 227, 283 321, 351 Mean (±SE)= 272±24	81, 75 34, 91 92, 111 Mean (±SE)= 81±11
CFU-GM	321, 299 309, 312 114, 121 90, 135 Mean (±SE)= 213±37	287, 360 316, 262 109, 103 Mean (±SE)= 240±44	321, 339 289, 324 84, 106 Mean (±SE)= 244±48	354, 319 349, 271 94, 103 Mean (±SE)= 248±49

As shown in Table 1, when A⁻T⁻MNC were employed as indicator cells, c-kit antisense oligomers inhibited growth of CFU-E ~75%, and BFU-E ~71%, when employed at the highest doses. Inhibition was sequence-specific since neither sense, nor scrambled sequence oligomers, significantly affected colony growth in comparison to untreated controls. In contrast to these results, CFU-GM and CFU-MEG derived colony formation was unaffected by exposure to any of the oligomers, at any of the doses employed (Table 1).

Similar results were obtained after exposure of $CD34^+$ cells to c-kit oligomers. As shown in Table 2, the mean number of CFU-E colonies decreased by ~66% after exposure to c-kit antisense oligomers while the number of BFU-E colonies decreased ~71%. Neither sense nor scrambled sequence oligomers inhibited colony formation. As was also noted with the less purified cell preparation, neither antisense nor control oligomers inhibited CFU-GM colony formation. The failure of c-kit antisense to inhibit CFU-GM and CFU-MEG is surprising since W/W^v mice have been reported to have defective granulopoiesis and megakaryocytopoiesis (Chervenick et al., Proc. Soc. Exp. Biol. Med. 152, 398-402 (1976)).

Erythroid colony formation was inhibited in a dose-dependent fashion. See Fig. 3, showing the effect of various concentrations of c-kit antisense oligomer on BFU-E-derived colony formation. Moreover, residual colonies were much smaller for the antisense-treated cells versus untreated controls (data not shown).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): As additional proof that the antisense effect was due to a specific decrement in c-kit mRNA levels, the kinetics of c-kit message expression in marrow mononuclear cells were examined, and the effect of oligomer exposure on c-kit mRNA levels was then assessed by the following RT-PCR procedure.

Total RNA was extracted from cells using the quanidine isothiocyante method of Chirgwin et al., Biochemistry, 18, 5294 (1979). Cells (2-5 x 10^6) were lysed in 250 µl of guanidine thiocyanate buffer (4M guanidine thiocyanate; 50 mM sodium acetate pH 5.0; 1 mM EDTA; 1M β-mercaptoethanol; 0.5% sarcosyl) and then layered over 250 µl of a cesium chloride (5.7 M) cushion in Beckman open-top ultra clear centrifuge tubes (0.8 ml). Tubes were centrifuged (Beckman TL-100 Ultracentrifuge; 100,000 RMP; 1.5 hours; 20°C) and the resulting RNA pellet was resuspended in ~400 µl of water, precipitated with 0.3 M potassium acetate, washed twice in 75% ethanol, and then stored at -70°C until used.

RNA was reverse-transcribed with 500 U of Moloney murine leukemia virus reverse transcriptase (MoMLV-RT) and 50 pmol of a 22-nucleotide oligodeoxynucleotide 3′ primer complementary to nucleotides 1201-1179 (CTAGG-AATGT GTAAGTGCCT CC, SEQ ID NO:16) of the published c-kit cDNA sequence. The resulting cDNA fragment was amplified using 5 U of Thermus aquaticus (Taq) polymerase and a 22-nucleotide oligodeoxynucleotide 5′ primer specific for c-kit nucleotides 842-864 (GGTTGACTAT CAGTTCA-GCG AG, SEQ ID NO:17). Twenty-five µl of amplified product was electrophoresed on 4% agarose gel and subsequently transferred to a nylon filter. Filters were pre-hybridized, and then probed with a ^{32}P end-labeled oligonucleotide probe (Caracciolo et al., Science 245, 1107-1109 (1989)) corresponding to the 21-nucleotide c-kit sequence (GATCCACTGC TGGTGTTCAG G, SEQ ID NO:18) contained within the amplified region (nucleotides 1068-1047). Autoradiography was performed by exposing filters on Kodak X-ray film at -70°C using intensifying screens.

A^-T^-MNC cells were kept for 24 hours at 4°C in IMDM containing 2% human AB serum, then shifted to 37°C and stimulated with IL-3 (20 U/ml) and EPO (5 U/ml) in 5% human AB serum. C-kit expression was determined at

intervals according to RT-PCR. The results are indicated in Figure 1. The Figure 1 lanes indicate relative c-kit transcript amounts determined by autoradiography of RT-PCR hybridization gels at the following intervals after stimulation:

Fig. 1 Lane	Time to RT-PCR Assay (hrs)
1	0
2	2
3	8
4	12
5	24
6	36
7	48
9	H_2O

Lane 8 contained H_2O as a control. Expression appeared to peak at ~36 hours.

A$^-$T$^-$MNC cells stimulated (t=0) in the same manner were exposed to c-kit sense, antisense or mismatch oligomers. c-kit expression was assayed by RT-PCR (t=36 hrs) as above. The results appear in Figure 2. The lanes are identified as follows:

Fig.2 Lane	Treatment
1	control cells (t=0)
2	control cells (t=36 hrs)
3	sense (t=36 hrs)
4	antisense (t=36 hrs)
5	mismatch (t=36 hrs)

Antisense-treated cells (lane 4) had no detectable c-kit mRNA, while sense (lane 3) and scrambled sequence (lane 5) treated cells had levels which were similar to those observed in untreated control cells at the same time point (lane 2).

Example 2

Effect of c-kit Antisense Oligomer Exposure on Malignant Hematopoietic Progenitor Cell Growth

To explore the importance of the c-kit receptor in regulating malignant hematopoietic cell growth, we employed a strategy which we have successfully employed in the past (Calabretta et al., Proc. Nat. Acad. Sci. USA, 88, 2351 (1991)), and which is essentially identical to that described above. A$^-$T$^-$MNC were obtained from patients with a variety of hematologic malignancies and exposed to the c-kit oligomers utilized in the preceding normal cell studies. Effects on the ability of malignant CFU-GM to form colonies, an index of the effect on malignant cell survival and proliferative activity, was then assessed. A total of twenty-two patients were studied, three with acute lymphocytic leukemia, four with acute myelogenous leukemia, ten with chronic myelogenous leukemia, and one with a myelodysplastic (preleukemic) syndrome and four with polycythemia vera. Overall response rates, and response rate by disease type, is given in Table 3.

Table 3

Effect of c-kit oligomers on growth of malignant hematopoietic colony forming cells

Disease Type	No. Pts	Responders	% Decrease Colonies	Non-Responders
Acute lymphocytic leukemia	3	1	68%	2
Acute myelogenous leukemia	4	1	63%	3
Chronic myelogenous leukemia	10	5	84% 33% 40% 90% 78% Mean=65%±26%	5
Myelodysplastic syndrome	1	1	68%	-
Polycythemia vera	4	4	Mean 74%±24%	-
TOTAL	22	12	66%	10

While the number of patients in categories other than CML is small, the data nonetheless suggest that patients with CML are particularly likely to respond to c-kit antisense. Accordingly, c-kit antisense oligomers are believed particularly useful as CML bone marrow purging agents. In addition, because of their marked inhibition of erythroid progenitor cells, it is believed that c-kit oligomers are useful in controlling the markedly elevated hemoglobin/hematocrit found in patients with PV, another myeloproliferative disorder.

Example 3

Effect of c-kit Antisense Oligomer Exposure on BFU-E Responsiveness to Stem Cell Factor

To provide proof that c-kit antisense mediated inhibition of erythropoiesis was due to the absense of KIT receptor function, we sought to demonstrate that BFU-E responsiveness to stem cell factor (SCF) could be abolished in a sequence-specified manner after exposure to c-kit oligomers. Accordingly, $CD34^+$ MNC (2×10^4) were cloned in the presence of 5 units of EPO and 100 ng of SCF per ml alone or with sense, antisense, or scrambled-sequence c-kit oligomers (final concentration, 150 μg/ml (~26 μM)). In four experiments, 191 ± 19 BFU-E (mean ± SD) were grown in the presence of the growth factors alone. These numbers were not statistically different from those cloned with sense (183 ± 29; P = 0.654) or scrambled-sequence oligomers (180 ± 20; P = 0.758). In the presence of the c-kit antisense oligomers, BFU-E-derived colony formation was completely abolished (0.4 ± 0.7; P<0.0001), suggesting that KIT receptor was no longer present to interact with its ligand.

The following non-limiting example illustrates one methodology for bone marrow purging according to the present invention.

Example 4

Bone Marrow Purging with c-kit Antisense Oligonucleotide

Bone marrow is harvested from the iliac bones of a donor under general anesthesia in an operating room using standard techniques. Multiple aspirations are taken into heparinized syringes. Sufficient marrow is withdrawn so that the marrow recipient will be able to receive about 4×10^8 to about 8×10^8 processed marrow

cells per kg of body weight. Thus, about 750 to 1000 ml of marrow is withdrawn. The aspirated marrow is transferred immediately into a transport medium (TC-199, Gibco, Grand Island, New York) containing 10,000 units of preservative-free heparin per 100 ml of medium. The aspirated marrow is filtered through three progressively finer meshes until a single cell suspension results, i.e., a suspension devoid of cellular aggregates, debris and bone particles. The filtered marrow is then processed further into an automated cell separator (e.g., Cobe 2991 Cell Processor) which prepares a "buffy coat" product, (i.e., leukocytes devoid of red cells and platelets). The buffy coat preparation is then placed in a transfer pack for further processing and storage. It may be stored until purging in liquid nitrogen using standard procedures. Alternatively, purging can be carried out immediately, then the purged marrow may be stored frozen in liquid nitrogen until it is ready for transplantation.

The purging procedure may be carried out as follows. Cells in the buffy coat preparation are adjusted to a cell concentration of about 2 x 10^7/ml in TC-199 containing about 20% autologous plasma. C-kit antisense oligodeoxynucleotide, for example, in a concentration of about 8 mg/ml, is added to the transfer packs containing the cell suspension. Recombinant human hematopoietic growth factors, e.g., rH IL-3 or rH GM-CSF, may be added to the suspension to stimulate growth of hematopoietic neoplasms and thereby increase their sensitivity c-kit antisense oligonucleotide toxicity. The transfer packs are then placed in a 37°C waterbath and incubated for 18 - 24 hours with gentle shaking. The cells may then either be frozen in liquid nitrogen or washed once at 4°C in TC-199 containing about 20% autologous plasma to remove unincorporated oligomer. Washed cells are then infused into the recipient. Care must be taken to work under sterile conditions wherever possible

and to maintain scrupulous aseptic techniques at all times.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

All references cited herein with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

SEQUENCE LISTING

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(B) **REGISTRATION NUMBER:** 30,480

(C) **REFERENCE/DOCKET NUMBER:** 6056-129 PC

(ix) **TELECOMMUNICATION INFORMATION:**

(A) **TELEPHONE:** (215) 568-8383

(B) **TELEFAX:** (215) 568-5549

(C) **TELEX:** None

(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 40 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:1:**

GAACGCAGAG AAAATCCCAG GCGCCGCGAG CGCCTCTCAT 40

(2) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 26 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:2:**

TCCCAGGCGC CGCGAGCGCC TCTCAT 26

(2) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 25 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:3:**

CCCAGGCGCC GCGAGCGCCT CTCAT 25

(2) **INFORMATION FOR SEQ ID NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 24 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:4:**

CCAGGCGCCG CGAGCGCCTC TCAT 24

(2) **INFORMATION FOR SEQ ID NO:5:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 23 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:5:**
CAGGCGCCGC GAGCGCCTCT CAT 23

(2) **INFORMATION FOR SEQ ID NO:6:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 22 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:6:**
AGGCGCCGCG AGCGCCTCTC AT 22

(2) **INFORMATION FOR SEQ ID NO:7:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 21 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:7:**
GGCGCCGCGA GCGCCTCTCA T 21

(2) **INFORMATION FOR SEQ ID NO:8:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 20 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:8:**
GCGCCGCGAG CGCCTCTCAT 20

(2) **INFORMATION FOR SEQ ID NO:9:**
(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 19 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:9:**
CGCCGCGAGC GCCTCTCAT 19

(2) **INFORMATION FOR SEQ ID NO:10:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 18 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:10:**
GCCGCGAGCG CCTCTCAT 18

(2) **INFORMATION FOR SEQ ID NO:11:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 17 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:11:**
CCGCGAGCGC CTCTCAT 17

(2) **INFORMATION FOR SEQ ID NO:12:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 16 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:12:**
CGCGAGCGCC TCTCAT 16

(2) **INFORMATION FOR SEQ ID NO:13:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 15 Nucleotides

(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:13:**
GCGAGCGCCT CTCAT 15

(2) **INFORMATION FOR SEQ ID NO:14:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 18 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:14:**
ATGAGAGGCG CTCGCGGC 18

(2) **INFORMATION FOR SEQ ID NO:15:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 18 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:15:**
GCACCGTCTG CCAGTCGC 18

(2) **INFORMATION FOR SEQ ID NO:16:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 22 Nucleotides
(B) **TYPE:** nucleic acid
(C) **STRANDEDNESS:** single stranded
(D) **TOPOLOGY:** linear
(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:16:**
CTAGGAATGT GTAAGTGCCT CC 22

(2) **INFORMATION FOR SEQ ID NO:17:**
(i) **SEQUENCE CHARACTERISTICS:**
(A) **LENGTH:** 22 Nucleotides
(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:17:**

GGTTGACTAT CAGTTCAGCG AG 22

(2) **INFORMATION FOR SEQ ID NO:18:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 21 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:18:**

GATCCACTGC TGGTGTTCAG G 21

CLAIMS

1. A pharmaceutical composition comprising a pharmaceutical carrier and an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

2. A composition according to claim 1 wherein the oligonucleotide comprises an at least 12-mer.

3. A composition according to claim 2 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

4. A composition according to claim 2 wherein the oligonucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

5. A composition according to claim 2 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

6. A composition according to claim 5 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleotide or a phosphorotioate oligodeoxynucleotide.

7. A composition according to claim 5 wherein the oligodeoxynucleotide comprises from a 15-mer to a 30-mer.

8. A composition according to claim 7 wherein the oligodeoxynucleotide comprises from an 18-mer to a 26-mer.

9. A composition according to claim 8 wherein the oligodeoxynucleotide comprises from an 18-mer to a 21-mer.

10. A composition according to claim 7 wherein the oligodeoxynucleotide has a sequence selected from the group of sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO:13.

11. A composition according to claim 10 wherein the oligodeoxynucleotide has a nucleotide sequence of SEQ ID NO:10.

12. An oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

13. An oligonucleotide according to claim 12 which comprises an at least 12-mer.

14. An oligonucleotide according to claim 13 having a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

15. An oligodeoxynucleotide according to claim 13 which is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

16. An oligodeoxynucleotide according to claim 13 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

17. An oligodeoxynucleotide according to claim 16 which is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

18. An oligodeoxynucleotide according to claim 16 which comprises from a 15-mer to a 30-mer.

19. An oligdeoxyonucleotide according to claim 18 which comprises from a 18-mer to a 26-mer.

20. An oligodeoxynucleotide according to claim 19 which comprises from a 18-mer to a 21-mer.

21. An oligodeoxynucleotide according to claim 16 selected from the group of oligodeoxynucleotides having sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and

SEQ ID NO: 13.

22. An oligodeoxynucleotide according to claim 21 having the nucleotide sequence SEQ ID NO:10.

23. A method for in vivo or ex vivo treatment of hematologic neoplasms characterized by c-kit expression comprising administering to a host in need of such treatment, or to cells harvested from such host, an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

24. The method according to claim 23 wherein the oligonucleotide is an at least 12-mer.

25. A method according to claim 24 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

26. A method according to claim 24 wherein the oligodeoxynucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

27. A method according to claim 24 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

28. A method according to claim 27 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

29. A method according to claim 27 wherein the oligodeoxynucleotide is selected from the group of oligodeoxynucleotides having sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,
SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO: 13.

30. A method according to any of claims 23, 24, 25, 26, 27, 28 or 29 comprising treating aspirated bone marrow cells and returning the aspirated cells to the host following treatment.

31. A method according to claim 23 wherein the hematologic neoplasm comprises chronic myelogenous leukemia.

32. A method according to claim 23 wherein the hematologic neoplasm comprises acute myelogenous leukemia.

33. A method for inhibiting proliferation of erythroid cells comprising administering to a host an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

34. A method according to claim 33 wherein the oligonucleotide is an at least 12-mer.

35. A method according to claim 34 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

36. A method according to claim 34 wherein the oligonucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion of the c-kit mRNA transcript including the translation initiation codon of said transcript.

37. A method according to claim 34 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

38. A method according to claim 37 wherein the oligodeoxynucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

39. A method according to claim 37 wherein the oligodeoxynucleotide is from a 15-mer to a 30-mer.

40. A method according to claim 39 wherein the oligodeoxynucleotide is from an 18-mer to a 26-mer.

41. A method according to claim 40 wherein the oligodeoxynucleotide is from an 18-mer to a 21-mer.

42. A method according to claim 41 wherein the oligodeoxynucleotide has a nucleotide sequence selected from the group of sequences consisting of:

SEQ ID NO:2,
SEQ ID NO:3,

SEQ ID NO:4,
SEQ ID NO:5,
SEQ ID NO:6,
SEQ ID NO:7,
SEQ ID NO:8,
SEQ ID NO:9,
SEQ ID NO:10,
SEQ ID NO:11,
SEQ ID NO:12, and
SEQ ID NO: 13.

43. A method for treating malignant melanoma comprising administering to a host in need thereof an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

44. A method according to claim 43 wherein the oligonucleotide comprises an at least 12-mer.

45. A method according to claim 44 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

46. A method according to claim 45 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

47. A method according to claim 45 wherein the oligonucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

48. A method for treating testicular or ovarian tumors comprising administering to a host in need thereof an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human c-kit gene, said oligonucleotide being hybridizable to said mRNA transcript.

49. A method according to claim 48 wherein the oligonucleotide comprises an at least 12-mer.

50. A method according to claim 49 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

51. A method according to claim 50 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the c-kit mRNA lying within about 40 nucleotides of the translation initiation codon.

52. A method according to claim 50 wherein the oligonucleotide is an alkylphosphonate oligodeoxynucleoside or a phosphorothioate oligodeoxynucleotide.

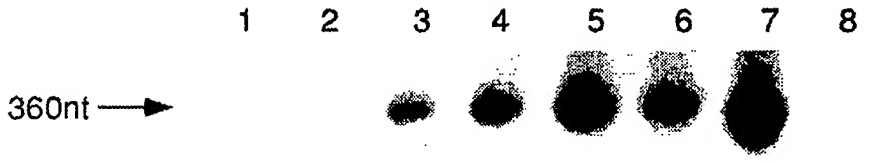

FIG. 1

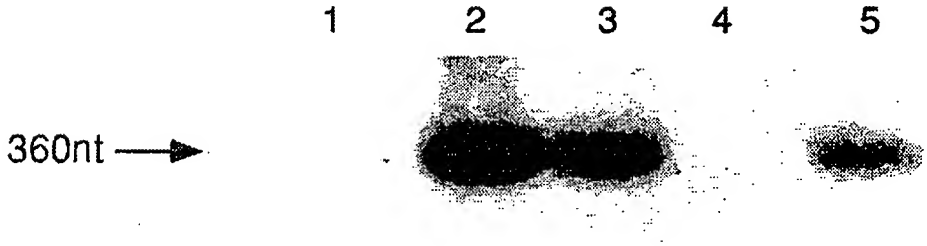

FIG. 2

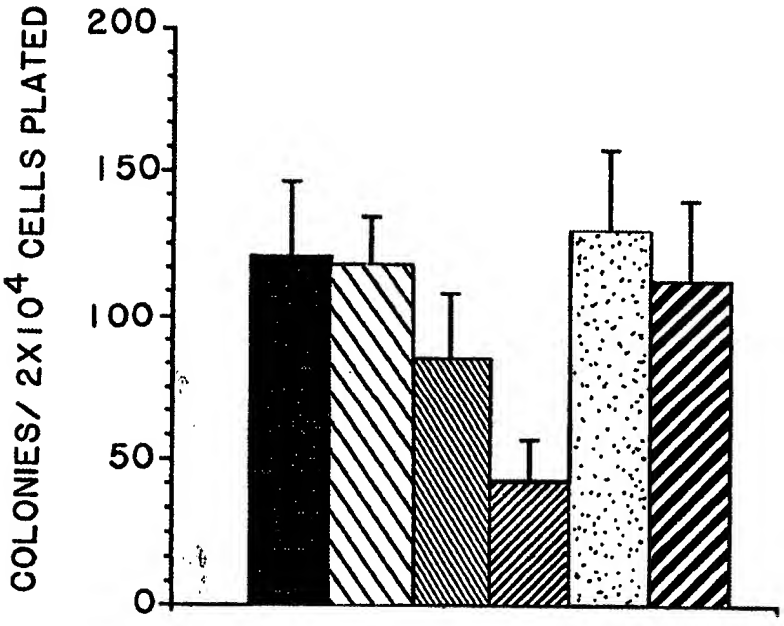

FIG. 3

INTERNATIONAL SEARCH REPORT

Int...national application No.
PCT/US92/02854

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 37/00, 31/70; C07H 15/12
US CL :424/93U; 514/44, 908; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93U; 514/44, 908; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO Journal, Volume 7,Number 4, issued 1988, F. Qiu et al. "Primary Structure of c-kit: Relationship with the CSF-1/PDGF Receptor Kinase Family - Oncogenic Activation of v-kit involves deletion of Extracellular Domain and C Terminus", pages 1003-1011, especially figure 2.	1-52
X	Cancer Research, Volume 51, issued 01 April 1991, T. Strohmeyer et al, "Expression of the hst-1 and c-kit Protooncogenes in Human Testicular Germ Cell Tumors", pages 1811-1816, especially "Results" pages 1812-1815, entire document.	1-52
X	Journal of Immunology, Volume 140, Number 7, issued 01 April 1988, D. Harel-Bellan et al, "Specific Inhibition of c-myc Protein Biosynthesis using an Antisense Synthetic Deoxy-Oligonucleotide in Human T Lymphocytes", pages 2431-2435, entire document.	1-52

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 August 1992	Date of mailing of the international search report 19 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DEBORAH CROUCH, PH.D. Telephone No. (703) 308-1126

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